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Assembly of RecA onto single-stranded DNA: Distinct role of mediator proteins in DNA repair and genetic recombination in *Bacillus subtilis*.

Doctoral Thesis

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DEDICATED TO MY BELOVED PARENTS

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Summary:

Natural competence is a programmed stage where inducible DNA uptake machinery localizes at cell pole of a differentiated bacterium. The DNA uptake machinery processes the environmental DNA and translocates ssDNA inside the bacterium. The cytosolic single-stranded binding, SSB (or Ssb), proteins acting as guardian and protect the incoming single-strand DNA (ssDNA), and at the same time limit RecA nucleation and filament growth onto the internalized ssDNA. SsbA, which is essential, binds ssDNA with 8-fold higher affinity than SsbB, suggesting that SsbA rather than SsbB might coat the internalized ssDNA. Although SsbB out numbers SsbA by 6 to 1.

Crystal study of SsbB bound to ssDNA shows that the protein has structural similarity with other SSBs, although is 33% smaller than the essential SSB proteins. The tetrameric SsbB binds ssDNA in two different forms, in the SSB_{35} mode only two protomers are wrapped by the ssDNA, and in the SSB_{65} mode the four protomers are wrapped by the ssDNA.

RecA function in recombination as higher-order oligomers assembled on tracts of ssDNA. These studies have identified a class of proteins, called recombination mediators, that act by promoting assembly of RecA onto SSB-coated ssDNA. In the absence of mediators, SsbA (or SsbB) inhibits RecA-mediated recombination by competing for binding to ssDNA. Inactivation of RecO and DprA leads ~1000-fold reduction in chromosomal transformation, suggesting that they might work as mediators. The mediator proteins have two activities: i) alleviate the restriction barrier imposed by SSBs proteins for RecA assembly onto ssDNA, and ii) catalyze single-strand annealing. The first activity is essential for *in vivo* function of RecA during DNA repair and chromosomal transformation; and the second is believed to be required for ssDNA-annealing during plasmid transformation, and perhaps for second end-capture during double strand break repair, although have not been clearly demonstrated.

In the presence of dATP, RecO alone does not significantly facilitate RecA filament growth onto naked ssDNA. RecO stimulates RecA assembly and DNA strand exchange onto SsbA·ssDNA or SsbA·ssDNA·SsbB complexes, and enhances DNA strand exchange. RecO fails to facilitate RecA growth onto SsbB, SSbB* (chimeric protein having full length SsbB and extreme C-terminal of SsbA) and SSB_{SPP1}. In the presence of ATP, RecO facilitates RecA assembly onto ssDNA, but it fails to promote RecA-mediated recombination. RecO facilitates RecA assembly onto SsbA- or SsbA·ssDNA·SsbB pre-coated ssDNA, and facilitates RecA-mediated DNA strand exchange.

DprA acts as a mediator protein. In the presence of dATP, DprA does not significantly facilitate RecA assembly onto ssDNA. DprA alleviates the SsbB barrier on RecA filament growth. RecA can assemble on the SsbB·ssDNA·DprA complex more efficiently than on SsbA·ssDNA·DprA complexes.

Inactivation of RecO or DprA reduces plasmid transformation by 30- or 40-fold respectively. However inactivation of both RecO and DprA strongly reduce plasmid transformation (~1000-fold). Mediator proteins bear annealing potential for complementary ssDNAs. The SsbA and SsbB protein limits annealing of complementary DNA strands. A mediator protein by dislodging its cognate SSB from ssDNA increases the spontaneous annealing of two complementary strands by a bridging mechanism. RecO interacts physically with SsbA and DprA with SsbB. RecO increases annealing of complementary strands coated by SsbA or SsbA and SsbB complex. On the other hand, DprA facilitates annealing of complementary strands coated by SsbB or SsbB and SsbA.

The absence of RecA, suppresses RecO for plasmid transformation, whereas the recA dprA double mutant strain decreases plasmid transformation by ~ 200-fold. Hence in the absence of RecA, DprA protein plays key role in plasmid transformation and work preferentially. Our data reveal a division of labor between SsbB and SsbA and the RecA mediators (DprA and RecO). RecO and DprA have specificity for SsbA and SsbB, respectively. The SSB proteins play an active role for the selection of effector proteins (DprA or RecO), for chromosomal and plasmid transformation.

Abbreviations:

DNA	Deoxyribonucleic acid
ssDNA	Single stranded DNA
dsDNA	Double stranded DNA
DNA pol	DNA polymerase
RNA	Ribonucleic acid
RNA Pol	RNA polymerase
BSA	Bovine serum albumin
BER	Base excision Repair
CCS	Circular single stranded DNA
PAGE	Polyacrylamide gel electrophoresis
DSB	Double stand DNA damage
DTT	Ditiotreitol
DSS	Disuccinimidyl suberate, Suberic acid
HR	Homologous recombination
KDa	Kilo-daltons
Kbp	Kilo-bases
LB	Luria-Bertani culture media
ldc	Linear double stranded DNA
Min	Minutes
MMC	Mitomycin C
MMS	Methyl Methanesulfonate
NER	Nucleotide excision repair
NHEJ	Non homologous end joining
nt	Nucleotide
pb	Base pair
PEI	Polyethyleneimine
AS	Ammonium sulphate
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in presence of SDS
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
C-ter	C- terminal
N- ter	N- terminal
SSA	Single stranded annealing
FD	Free DNA
Kd	Dissociation constant

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1. INTRODUCTION

1. Introduction:

Natural genetic transformation of bacteria encompasses the active uptake by a cell of free virus, plasmid or chromosomal DNA and the heritable incorporation of its genetic material, which contributes to the maintenance and evolution of bacteria. The process of natural transformation has been discovered by Griffith (1928) in *Streptococcus pneumoniae*, and later Avery and co-workers (Avery et al., 1944), have found for the first time that DNA was the main factor for transformation. Natural competent is widely spread in Bacteria, and more than 90 bacterial species have been shown to be naturally transformable (Stewart and Carlson 1986, Lorenz and Wackernagel 1994, Dubnau 1999, Claverys, Prudhomme et al. 2000).

Cellular competence is a programmed physiological state and induced by expression of proteins, which are mainly responsible for DNA uptake and processing, and the recombination machinery. In general there are three stages of DNA uptake and processing: binding to the environmental double-stranded (ds) DNA, degrading one strand to render single-stranded (ss) DNA, and transport it into the cytosol (Stewart and Carlson 1986, Smith, Tomb et al. 1995, Dubnau 1999, Claverys, Prudhomme et al. 2000, Chen and Dubnau 2004, Claverys, Martin et al. 2009). After DNA processing, the internalized ssDNA, if homology is provided, recombines with recipient, representing a form of bacterial sexual reproduction, which increases standing genetic variation of cell. Genetic recombination (GR) is the process by which the internalized ssDNA recombine with the homologous recipient (chromosomal transformation) or self-annealing (viral and plasmid transformation) and the product of the reaction gave to the cells a new phenotype. In the later process the incoming ssDNA, which shares no homology with recipient, but if it has a self-replication potential, can be reconstituted into its circular dsDNA form using recombination and replication function (Stewart and Carlson 1986, Viret, Bravo et al. 1991, Smith, Tomb et al. 1995, Dubnau 1999, Claverys, Prudhomme et al. 2000, Chen and Dubnau 2004, Claverys, Martin et al. 2009).

Natural competence is found in a broad spectrum of bacterial phyla. The most comprehensively studied species from Firmicutes phylum are *S. pneumoniae* and *Bacillus subtilis* and from Proteobacteria phylum are *Neisseria gonorrhoeae, Haemophilus influenza,* and *Helicobactor pylori* (Stewart and Carlson 1986, Smith, Tomb et al. 1995, Dubnau 1999, Claverys, Prudhomme et al. 2000). The occurrence of competence varies in bacterial species; it can be constitutively expressed or inducible and time-limited. Natural competence in *N. gonorrhoeae* is constitutively expressed (Dubnau 1999, Claverys, Prudhomme et al. 2000). Whereas inducible natural competence varies among bacteria and it is not regulated by available free dsDNA in the microenvironment, but other factors promote its induction. Competence may be induced in response to specific environmental conditions, such as in *B. subtilis*, that is caused by intrinsic noise in competence gene expression (bistability) (Solomon and Grossman 1996, Macfadyen 2000, Maamar and Dubnau 2005), by the presence of biofilms on chitin surfaces, like in *Vibrio cholera* (Dubnau 1999, Claverys, Prudhomme et al. 2000, Meibom, Blokesch et al. 2005, Claverys, Prudhomme et al. 2006, Dorer, Fero et al. 2010, Charpentier, Kay et al. 2011, Lo Scrudato and Blokesch 2012) or by the presence of antibiotics or DNA damage, as occurs in *S. pneumoniae, H. pylori,* and *Legionella pneumophila*. During competency, the metabolic activity of *B. subtilis* competent cells is reduced (Nester and Stocker 1963), cell division is inhibited and DNA replication is halted (Haijema, Hahn et al. 2001, Briley, Dorsey-Oresto et al. 2011) but the SOS system slightly induced.

DNA sources could vary for bacterial species, and most of the naturally competent bacteria, such as *B. subtilis*, *S. pneumonia, H. pylori, Pseudomonas stutzeri* and *Acinetobacter baylyi* can uptake DNA from any source (non selective uptake) (Dubnau 1999, Claverys, Prudhomme et al. 2000, Meier, Berndt et al. 2002), usually from their microenvironment where other closely related organisms grow. Whereas other bacteria, such as *N. gonorrhoeae* and *H. influenzae*, take up dsDNA only from their own clade (selective uptake) (Sisco and

Smith 1979, Mathis and Scocca 1982, Lorenz and Wackernagel 1994). The selective uptake of DNA depends on the presence of sequence-specific dsDNA binding protein, which allows efficient transport of a DNA that contains an uptake signal sequence (USS, also known as DNA uptake sequences, DUS) into the periplasm (Chen and Dubnau 2004). In all natural competent bacteria, DNA transformation is also controlled at several levels such as formation of a stable displacement loop (D-loop). In *B. subtilis*, a short segment of ~ 30-nt in length of identity is the minimum length required for RecA-mediated DNA strand invasion and D-loop formation (Majewski and Cohan 1999), but stability of the D-loops also depends on the RecA accessory factors. The uptake of DNA during natural competence is also affected by restriction and modification systems. The restriction and modification systems are abundant in bacteria, are traditionally associated with a cellular protection mechanism against incoming foreign dsDNA, mainly of bacteriophage origin. The up taken DNA, during competence, overcomes the host restriction and modification barriers because of its linear ssDNA nature. Therefore, the potential negative effect of restriction on interspecies transformation generally is not significant in naturally competent cells. Furthermore, in some bacterial species (e.g. *S. pneumoniae*) the methylation of the incoming ssDNA actively protects it from the endonuclease and allows transformation without any potential restriction (Lacks, Ayalew et al. 2000).

The 'quorum sensing' mechanism leads to up regulation of "early" competence (*com*) genes and induced natural competence in Firmicutes (Dubnau 1999, Morrison and Lee 2000, Lopez, Vlamakis et al. 2009, Shank and Kolter 2011). In *S. pneumoniae* competence induction occurs via two-component signal transduction system ComD-ComE to respond to extracellular peptide(s) ComX and competence-stimulating peptide (CSP). However in *B. subtilis* competence induction occurs in similar fashion as *S. pneumoniae* with involvement of ComP-ComA two-component signal transduction system, ComX and then competence and sporulation factor (CSF) (Dubnau 1999, Mirouze, Berge et al. 2013) The DNA uptake machinery does not have ability to distinguish between linear chromosomal DNA from super coiled plasmid DNA (Stewart and Carlson 1986, Dubnau 1999), and both compete for receptor sites at the cell surface (Gromkova and Goodgal 1981).

1. 1. DNA uptake and recombination machineries localization:

The transport of DNA from the environment into the cytosol of the bacteria is very critical step of the uptake process. The DNA transport through cytosolic compartment requires energy to drive through thus it is an energy-demanding task. In *B. subtilis* and *H. pylori* at least 16 competence-specific (Com) proteins has been shown to be localized at the cell pole (Chen, Christie et al. 2005, Kidane, Carrasco et al. 2009, Stingl, Muller et al. 2010).

In *B. subtilis,* based on their function, the cell pole localized proteins generally work at three different levels. The first group participates in a sequential step of DNA recognition, binding and processing from the environmental dsDNA. This group of protein includes those ones that form the competence pseudo-pilus (ComG proteins), which facilitates the pulling of DNA towards the translocation machinery in the cytoplasmic membrane, and the NucA endonuclease, which generates double strand breaks (DSBs) (Figure 1). One strand of internalized dsDNA undergoes fragmentation process by an unknown enzyme, which subsequently degraded into the extracellular environment. The ComG proteins are groups of several membrane bound proteins with different functions. It includes the ComGA ATPase, ComGB polytopic membrane protein, ComGC major pre-pilin–like protein, and three minorpre-pilin proteins ComGD, ComGE, and ComGG (Figures 1 and 2). The pre-pilin proteins integrate into the cytoplasmic membrane, and when processed by the peptidase ComC, these subunits translocate to the exterior of the membrane (Albano, Breitling et al. 1989, Puyet, Greenberg et al. 1990, Chung and Dubnau 1995, Chung, Breidt et al. 1998, Provvedi, Chen et al. 2001, Chen and Dubnau 2004, Chen, Provvedi et al. 2006, Craig and Li 2008, Briley, Dorsey-Oresto et al. 2011). The second group of proteins are generally known as transporter proteins and responsible for the transport of ssDNA crossways the cell membrane into the cytosol of the bacterial cell. This group includes ComFA, ComEA, and

ComEC proteins, which form the membrane transport apparatus (Figures 1 and 2) (Londono-Vallejo and Dubnau 1993, Inamine and Dubnau 1995, Provvedi and Dubnau 1999, Meima, Eschevins et al. 2002, Draskovic and Dubnau 2005, Kaufenstein, van der Laan et al. 2011).

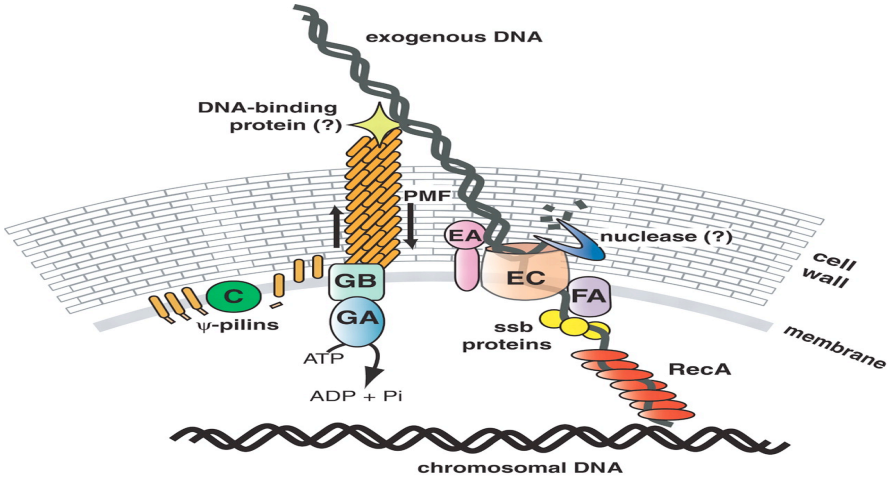


Figure 1. DNA uptake during transformation in *B. subtilis*. The uptake machinery is preferentially located at the cell poles. The Ψ-prepilins are processed by the peptidase and translocate to the outer face of the membrane. With the aid of the other components, the major Ψ -pilin ComGC assembles into the Ψ -pilus, which attaches exogenous DNA via a hypothetical DNA binding protein. Retraction of the Ψ -pilus, driven by the proton motive force, and DNA binding to the receptor (ComEA) are required to transport one strand of DNA through the membrane channel (ComEC) while the other is degraded by an unidentified nuclease. The helicase/DNA translocase (ComFA) assists the process, along with ssDNA binding proteins that interact with the incoming DNA. RecA, with the aid of accessory proteins, forms a filament around the ssDNA, and mediates a search for homology with chromosomal DNA (Chen, Christie et al. 2005).

The ComFB, ComFC, and ComEB, of unknown function, also co-assembled with the polar uptake machinery (Kaufenstein, van der Laan et al. 2011) ComGA (first group) and ComFA (second group) coordinate the interaction of the DNA uptake apparatus with proteins of the recombination machinery (third group) (Figure 2) (Kramer, Hahn et al. 2007). The third group proteins participate in the recombination machinery. Some of them transiently and specifically localize at the cell membrane prior to the internalization of ssDNA. The polar-localized recombination machinery, which protects, processes, and recombines the incoming ssDNA, includes five competence-induced (DprA, SsbA, SsbB, CoiA, and RecA) and at least two competence-independent (RecU, RecX) proteins (Figure 2). In the presence of external DNA another set of proteins selectively localizes at the cells pole (e.g., RecN, RecO) (Figure 2). ComGA, ComFA, and SsbB, which localize at the cell pole in rod-shaped bacteria, appear to promote the localization of CoiA, DprA, and RecA. In some cases the assembly might occur away from the pole and these complexes diffuse until they are captured at the pole (Hahn, Kramer et al. 2009, Kaufenstein, van der Laan et al. 2011). For example, cytosolic SsbB can associate with the pole independently of other Com proteins and its absence does not seem to affect ComGA and RecA localization, but decreased DprA localization (Kramer, Hahn et al. 2007).

During the phase of natural competence some other cellular processes also affect localization and delocalization of membrane bound and cytosolic proteins. Such as post-translational modifications, which phosphorylate arginine (e.g. ClpC, ClpP, McsB, ComGA, ComFA, ComFC, RecA, SsbA, and SsbB) and/or tyrosine (SsbA, SsbB) residues (Mijakovic, Petranovic et al. 2006, Elsholz, Turgay et al. 2012), resulted in changed localization. The mechanisms for this phosphorylation effect on protein for localization is not very clear and remain undefined.

In the case of Proteobacteria (eg. *N. gonorrhoeae* and *H. pylori*), which have two membranes so that the DNA uptake machinery needed to cross another membrane. In Proteobacteria, the dsDNA is bound on the cell surface and transported as intact dsDNA into the periplasm through a ring shaped assembly of proteins of a type IV pili known as secretins (e.g. PilQNgo) (Mattick 2002, Collins, Frye et al. 2004), or through a type IV secretion

system as the one used to transport dsDNA into the periplasmic space in *H. pylori* (Hofreuter, Odenbreit et al. 2001). Once dsDNA has crossed the outer membrane and is in the periplasmic region of the cell, the transport of the ssDNA into the cytosol is based on the DNA uptake machinery, similar to the one described for Firmicutes (Danner, Deich et al. 1980, Elkins, Thomas et al. 1991, Stingl, Muller et al. 2010).

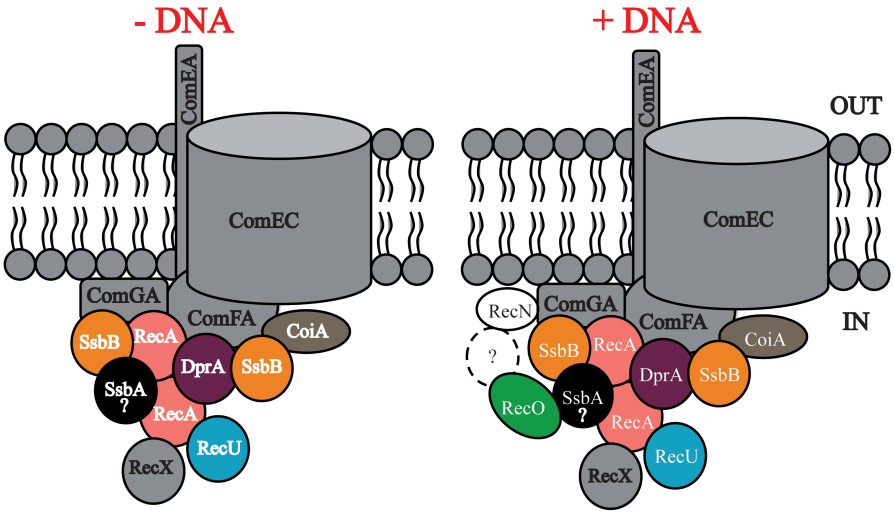


Figure 2: Differential protein localization at the cell pole in the absence (- DNA) and the presence (+ DNA) of any source during programmed natural transformation

In some other cases the protein associated with the cell pole (e.g. ComGA) facilitates the localization of a cytosolic protein (e.g. RecA), and further facilitates the localization of other cytosolic protein (e.g. DprA, RecU) (Figure 2). However, DprA, which forms polar assembly in the absence of RecA, is loosely associated with other uptake proteins (e.g. SsbB, RecA) (Kidane and Graumann 2005, Kramer, Hahn et al. 2007, Hahn, Kramer et al. 2009). RecU, which is a RecA modulator and a Holliday junction [HJ] resolving enzyme (Ayora, Carrasco et al. 2004, McGregor, Ayora et al. 2005)) also localizes at pole but only in the presence of RecA (Kidane, Carrasco et al. 2009). In general polar assembly of all protein occurs at a single cell pole except few cases where assembly at both poles was also observed (Hahn, Maier et al. 2005). This suggest us that: i) the DNA uptake complex is dynamic, ii) occurrence of all polar localized proteins might not co-exist simultaneously or their stoichiometry might vary, and iii) in general one but rarely two uptake machineries per cell might be present for DNA transfer. The RecN (or SbcE in the absence of RecN) dynamically oscillate from one cell pole to another cell pole for the scanning of incoming ssDNA. The RecN or SbcE belong to the family of structural maintenance of chromosomes (SMC)-like proteins (Graumann and Knust 2009). However when ssDNA is inside the cell then RecN (or SbcE) loses its dynamic oscillation and binds to the incoming DNA ends (Figure 2) (Kidane and Graumann 2005, Krishnamurthy, Tadesse et al. 2010). The internalization of ssDNA segments with self-annealing potential (e.g. during plasmid transformation) promotes the localization of RecO, which is modulated by the presence of RecN (Figure 2) (Kidane, Carrasco et al. 2009).

1. 2. Fate of internalized ssDNA:

After internalization of ssDNA inside the cell it can undergo two processes depending on homology availability with the recipient genome:

(A) When the incoming ssDNA shows complementarities with recipient dsDNA, it recombines and integrates into the chromosome. It is an intermolecular recombination process that leads to the formation of a D-loop intermediate, due to the exchange of a strand of the recipient molecule with the newly internalized ssDNA molecule. Then, the D-loop is resolved, and the replaced strand degraded (Figure 3A). Chromosomal transformation is a

RecA protein dependent process (Fernandez, Ayora et al. 2000). The chromosomal transformation reaction is very efficient, and it does not require extensive DNA replication because less than 300-nt of *de novo* synthesis is sufficient for integration of the donor markers (Lorenz and Wackernagel 1994, Dubnau 1999). The three-stranded homologous recombination (HR) reaction, which takes place during natural transformation by recombination of the linear taken up ssDNA with recipient dsDNA is one of the most efficient ways for cells to acquire new genetic traits, because ~ 40% of internalized ssDNA becomes a recombinant product (Dubnau 1999, Fernandez, Ayora et al. 2000, Claverys, Martin et al. 2009).

(B) When the incoming ssDNA is an autonomous replication molecule that shows no homology with recipient genome, two different events might take place: plasmid transformation (if imported ssDNA molecule is oligomeric) (Figure 3B) or monomeric activation (if ssDNA contains only internal repeats) (Kidane et al 2012). Plasmid transformation and monomeric activation are intra-molecular recombination processes in which the internalized strand(s) recombines to reconstitute a circular dsDNA molecule. Depending on the type of plasmid substrate this could be RecA-independent process (plasmid transformation) or partially RecA-dependent (monomeric activation) (Canosi, Morelli et al. 1978, Michel, Niaudet et al. 1982). There is another complex recombination event called viral transfection, which is poorly understood. Viral transfection is an inter- and intra-molecular recombination process (Sanchez, Carrasco et al. 2006, Kidane, Carrasco et al. 2009).

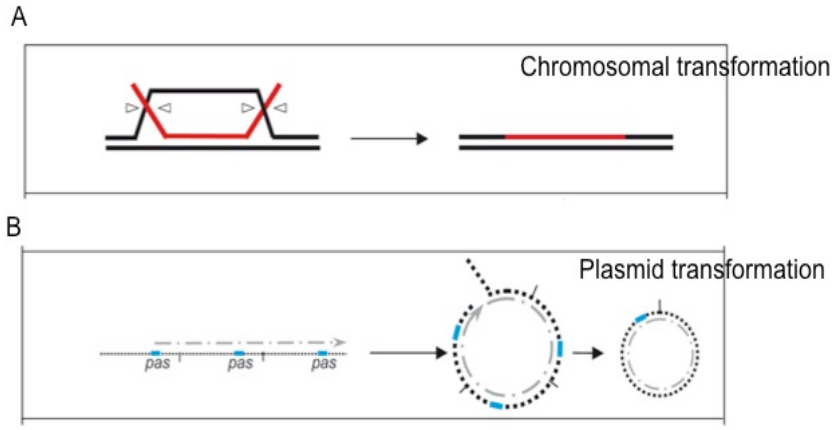


Figure 3: Fate of internalized ssDNA during genetic recombination (A) Chromosomal transformation (B) Plasmid transformation

Chromosomal DNA transformation efficiency is higher than that of plasmid transformation (~100-fold) (Canosi, Morelli et al. 1978, Dubnau, Contente et al. 1980). The monomeric plasmid DNA is not the proper substrate for naturally transforming bacteria because artificial oligomerization of plasmid DNA markedly increases plasmid transformation efficiency (Eisenstein, Sox et al. 1977, Canosi, Ferrari et al. 1979, Notani, Setlow et al. 1981). The competent cell interacts with a single plasmid molecule of DNA to deliver a plasmid transformant because dimeric or higher order oligomeric plasmid DNA follows a first order kinetics with respect to DNA concentration. It was hypothesized that the internalized ssDNA remains intact as long as RecA is searching for homology (chromosomal transformation) or a replication origin and internal homology is found (plasmid transformation), but it is degraded when none of those conditions are fulfilled (Dubnau and Cirigliano 1973, de la Campa, Springhorn et al. 1988).

1. 3. Genetic analysis of the recombination machinery:

Genetic analyses have allowed us to gain insight into the contribution of DNA repair and competence-specific functions in chromosomal and plasmid transformation. Note that henceforward in this thesis and unless stated otherwise, the indicated genes and their products are of *B. subtilis* origin. The nomenclature used to denote the origin of proteins from other bacteria is based on the bacterial genus and species (e.g., *S. pneumoniae* RecA is referred to as $RecA_{Spn}$). *In vitro*, RecA has been shown to catalyze the formation of heteroduplex joints. In the absence of RecA, DNA repair and chromosomal transformation are absolutely blocked (Kidane et al 2012). The plasmid transformation, in general, is a RecA independent event (2- to 4-fold reduction), except in some species (e.g. *S. pneumoniae*) where plasmid transformation is indirectly abolished in the absence of RecA. In the absence of RecO, the plasmid transformation is affected very high (~ 30 fold reduction) than the chromosomal transformation (2-3 fold reduction).

DprA (DNA processing protein) is a ubiquitous protein. Two DprA family members (Smf and DprA) are present in *E. coli* cells. Here, Smf-mediated artificial transformation is independent of Ca^{2+}-mediated plasmid transformation or HFR conjugation in *E. coli* K12 cells (Smeets, Becker et al. 2006). Thus, in the case of non-natural competent bacteria, the role fulfilled by DprA/Smf, is remains to be unraveled. DprA plays a prominent role in natural competent cells. The absence of DprA reduced ~100-fold chromosomal transformation in *B. subtilis*, *H. influenzae*, *D. radiodurans,* and perhaps in *S. pneumoniae* (Karudapuram and Barcak 1997, Ando, Israel et al. 1999, Smeets, Bijlsma et al. 2000, Berka, Hahn et al. 2002, Hamoen, Smits et al. 2002, Ogura, Yamaguchi et al. 2002, Tadesse and Graumann 2007, Marsin, Mathieu et al. 2008, Bouthier de la Tour, Boisnard et al. 2011, Satoh, Kikuchi et al. 2012). The role of DprA in plasmid transformation is less uniform. Plasmid transformation decreases in *H. pylori*, *B. subtilis* and *S. pneumoniae* cells in the absence of DprA, but its absence does not seem to affect plasmid transformation in *H. influenzae* cells (Karudapuram and Barcak 1997, Ando, Israel et al. 1999, Smeets, Bijlsma et al. 2000, Berka, Hahn et al. 2002, Hamoen, Smits et al. 2002, Ogura, Yamaguchi et al. 2002, Tadesse and Graumann 2007, Marsin, Mathieu et al. 2008).

The absence of SsbB or CoiA, in both *B. subtilis* and *S. pneumoniae* competent cells, moderately reduces (3- to 20-fold) chromosomal transformation, suggesting that both proteins play a minor role in transformation, or that the redundancy of the system masks the outcome.

The *B. subtilis* recombinational repair proteins other than RecA, have been classified into nine different epistatic groups (α to η) (Ayora, Carrasco et al. 2011). The absence of RecX reduced chromosomal transformation ~ 200-fold in *B. subtilis*, but only 5-fold reduced in *N. gonorrhoeae* (Stohl and Seifert 2001, Cardenas, Carrasco et al. 2012). More than one pathway might act redundantly to promote chromosomal transformation (Alonso, Stiege et al. 1993), and it was shown that the absence of both pathways, e.g., RecF and AddAB (counterpart of the RecBCD enzyme), reduced natural transformation >500-fold.

The absence of RecX, RecO or RecU reduced plasmid transformation by ~50-, ~30-fold and ~40-fold, respectively (Fernandez, Sorokin et al. 1998, Fernández, Kobayashi et al. 1999, Kidane, Carrasco et al. 2009, Cardenas, Carrasco et al. 2012). In some other bacteria the requirement of recombination proteins is not universal for plasmid transformation. In case of *D. radiodurans* competent cells is not affected by the absence of RecO, but is reduced (>100-fold) in the absence of the RecR (RecO associate partner), and of another species specific single strand annealing (SSA) protein (e.g. DdrB) play a relevant role in plasmid transformation (Bouthier de la Tour, Boisnard et al. 2011, Satoh, Kikuchi et al. 2012).

1.4. Biochemical analysis of the recombination machinery in genetic recombination process:

1.4.1. RecA:

The RecA protein of *B. subtilis* is a 347-amino-acid polypeptide with a molecular mass (Mm) of 37,933 Da, and a pI of 4.88. RecA has at least three distinct functions in DNA metabolism: i) RecA filamented onto ssDNA activates the self-degradation of the LexA repressor and it regulates the induction of the SOS response to extensive DNA damage; ii) the RecA·ssDNA filament catalyzes DNA strand exchange, which is universally shared by the recombinases and it represents the central process in any homologous genetic recombination reaction; and iii) RecA·ssDNA participates directly in SOS mutagenesis (Cox 2003, Cox 2007).

The RecA structure reveals a central core domain and two smaller domains at the amino (N) and carboxyl (C) termini. The N-terminal domain appears to be involved primarily in monomer-monomer interactions. The core domain is structurally homologous to several proteins, including hexameric helicases, and the mitochondrial F1-ATPase. This domain is part of the protein that is most highly conserved among bacterial species. The RecA core domain features the ATP/ dATP binding site and the probable DNA binding sites. It has been reported that the RecA has high nucleotide hydrolysis activity in presence of dATP than the ATP as cofactor (Menetski and Kowalczykowski 1989). The C-terminal domain of the RecA protein exhibits little sequence conservation. The C-terminal domain movement relative to the core domain may be responsible for the "active" or "inactive" state of the RecA filament. In addition, this domain seems to shift position in response to identity of the nucleotide bound at the ATP binding site, which implies that the domain may move during ATP hydrolysis (Cox 2003).

Recombination reactions catalyzed by the RecA family of protein form an integral part of DNA metabolism in all free-living bacteria, archaea and eukaryotes (Cox 2007, San Filippo, Sung et al. 2008). The major role of RecA in competent cells is to form a helical nucleoprotein filament (visualized as dynamic threads structures across the nucleoid) on the internalized ssDNA and then promotes a search for homology with dsDNA of recipient DNA, leading to integration into the genome of the competent cell (Kidane and Graumann 2005, Sanchez, Carrasco et al. 2006). The assembly of RecA onto the incoming ssDNA is comparatively infrequent and thus rate limiting. RecA required a set of accessory factors to stimulate RecA nucleation and filament extension onto the incoming ssDNA. The accessory factors, which act before RecA nucleation, can be divided into two groups depending on functionality. The first group includes single-strand binding proteins (SSB) that act as protectors (SSB/SsbA, SsbB, RecN, and SbcE), barriers (SSB/SsbA, SsbB), and constrainers of RecA nucleation onto ssDNA (SSB/SsbA, SsbB. These proteins are called guardian proteins. Genetic and biochemical data support the hypothesis that among the "guardians", RecN or SbcE might protect the ends of the internalized linear ssDNA and SsbA and/or SsbB coat the incoming ssDNA and remove secondary structures that hamper the downstream reaction (Kidane and Graumann 2005, Carrasco, Manfredi et al. 2008, Krishnamurthy, Tadesse et al. 2010).

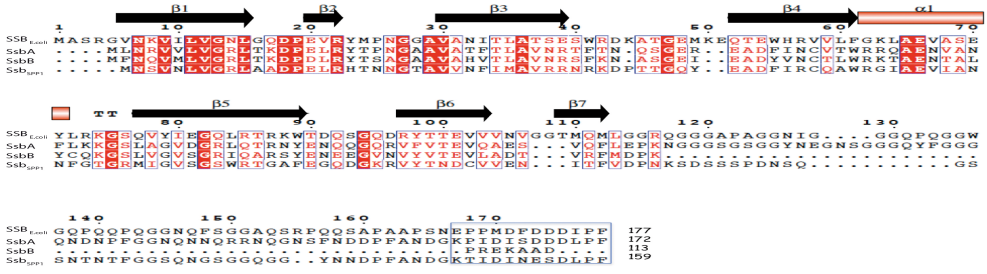
The second group includes proteins (RecO, DprA) that reduce barriers and promote the nucleation of RecA protein onto ssDNA and facilitate annealing of complementary DNA strands coated by SSBs. These groups of proteins are termed as mediator's proteins (Beernink and Morrical 1999, Cox 2007, Galletto and Kowalczykowski 2007). The RecA modulator proteins (RecF, RecX, RecU) are the accessory factors that act during RecA-mediated homology search and regulate the dynamic assembly/disassembly of the RecA·ssDNA filament.

1.4.2. Single stranded binding protein (SSB):

SSB proteins bind to ssDNA with high affinity and play critical roles as accessory proteins in DNA replication, recombination, and repair (Lohman and Ferrari 1994). In general there are two common structural features present in SSB proteins. The first is the oligonucleotide/oligosaccharide-binding (OB) domains, which is responsible for binding with ssDNA. This interaction occurs through a combination of electrostatic and base-stacking interactions with the phosphodiester backbone and nucleotide bases, respectively. The second feature is the SSB oligomerization domain that brings together four DNA-binding OB folds in the protein's active form (tetramer) (Figure 4). SSB_{Eco} has served as the prototypical SSB protein for decades, which encodes a single OB fold in each monomer and functions as a tetramer (Lohman and Ferrari 1994, Shereda, Kozlov et al. 2008).

SSB proteins can bind ssDNA in high affinity leading to clusters of SSB tracts to form bead-like necklace on long ssDNA. SSB tetramer can bind to long stretches of ssDNA in multiple binding modes differing in the number of OB-folds that interact with the ssDNA. The prime ssDNA binding modes are denoted as the $(SSB)_{65}$ and $(SSB)_{35}$ modes, where the subscript reflects the average number of nucleotide residues bound by each tetramer in the SSB-ssDNA complex. In the case of $(SSB)_{65}$ mode, ~ 65-nucleotides (nt) of ssDNA interact and wrap around with all four subunits of the tetramer. However in the $(SSB)_{35}$ mode, ~ 35-nt interact and wrap around with an average of only two subunits. The $(SSB)_{65}$ binding mode occurs when there is a limited co-operativity mode present and SSB shows little affinity to form protein clusters along ssDNA and wrap around with all four subunits of the tetramer. On the other hand the $(SSB)_{35}$ binding mode is a high, unlimited co-operativity mode in which SSB can form long protein clusters along ssDNA and wrap around with an average of only two subunits. The relative stabilities of these modes of SSB-ssDNA binding are influenced by several factors, like monovalent (NaCl) and divalent (Mg^{2+}) salt concentrations, as well as spermine, polyamines and spermidine concentrations (Lohman and Ferrari 1994, Shereda, Kozlov et al. 2008). It has been proposed that the different SSB binding modes may be used selectively in different processes in the cell, however the specific mode functioning *in vivo* is not clear (Lohman and Ferrari 1994, Shereda, Kozlov et al. 2008).

A)



B)

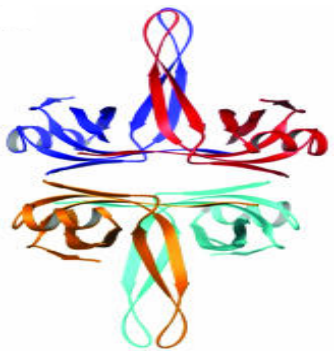


Figure 4: Sequence alignment and crystal structure of Single stranded binding protein from *E. coli*. (A) Sequence alignment of SSBs (B) Crystal structure of SSB as tetramer

With few exceptions (e.g. *H. pylori*, *D. radiodurans* and *Campylobacter jejuni*), naturally transformable bacteria contain two different types of SSBs, they are called as SsbA- and SsbB- like proteins (based on protein length) (Figure 4A), whereas only one single SsbA-like protein is present in the non-naturally transformable bacteria (Lindner, Nijland et al. 2004). In *B. subtilis* SsbA (counterpart of Escherichia coli SSB [SSB_{Eco}]) is a 172-residue polypeptide (Mm 18,598 Da; pI 4.82) that shares strong sequence similarity with the DNA-binding N-terminal domain (OB domain) and disordered C-terminus protein-binding domain of SSB_{Eco} (Figure 4B). SsbA is an essential homotetrameric protein involved in genome maintenance (Ayora, Carrasco et al. 2011). The expression of SsbA is also induced with the development of natural competence (Berka, Hahn et al. 2002, Ogura, Yamaguchi et al. 2002), but $SsbA_{Spn}$ is not induced during competence development (Attaiech, Olivier et al. 2011). SsbA physically interacts with RecO (Manfredi, Carrasco et al. 2008) as well as with many other recombination proteins. SsbA relative protein concentration and cellular localization in competent cells is not very well known, it might be possible that the protein is induced to levels similar to those of SsbB in a small subset, likely at amounts equal to or greater than levels of SsbA found during exponential growth (>750 tetramers per cell) (Berka, Hahn et al. 2002).

The SsbB protein is a relatively shorter polypeptide than the SsbA (Figure 4A). SsbB is 113-residue polypeptide (Mm 12,350 Da; pI- 6.88) that is specialized for activities in transformational recombination. SsbB is over-expressed during competence development. SsbB shares 63% identity with the N-terminal DNA binding domain of SsbA (amino acids 1–106), but lacks the characteristic C-terminal tail (Figure 4A). *In vivo* analyses in *B. subtilis* reveal that SsbB is located at the DNA entry poles in competent cells and is in contact or close proximity with RecA, CoiA and DprA (Figure 2) (Hahn, Maier et al. 2005, Kramer, Hahn et al. 2007). The absence of SsbB only moderately reduces chromosomal transformation (3- to 10-fold) in both *B. subtilis* and *S. pneumoniae* cells (Berka, Hahn et al. 2002, Ogura, Yamaguchi et al. 2002), suggesting other protein(s) might protect the internalized ssDNA. Unlike *B. subtilis* SsbB, $SsbB_{Spn}$ is ~20-fold more abundant than $SsbA_{Spn}$ (Attaiech, Olivier et al. 2011). The *B. subtilis* SsbB lacks the prototypical acidic C-terminal domain for protein interactions, but some naturally competent bacteria, e.g. *S. pneumoniae*, have SsbB proteins with this C-terminal tail that might serve for protein interactions (Attaiech, Olivier et al. 2011).

1.4.3. RecO:

The assembly of RecA onto the incoming ssDNA is comparatively infrequent and thus rate limiting. A SSB protein (e.g., Gp32, SSB/SsbA, RPA) bound onto ssDNA to protect it from degradation, and also exert a negative effect for RecA nucleation. A family of *mediator* proteins (e.g., viral UvsY, bacterial RecO(R), DprA or eukaryotic Rad52, BRCA2) has evolved to counteract the limitation exerted by their cognate SSB. Mediator proteins not only work to counteract the recombinase limitation, but it also catalyzes ssDNA strand annealing. For example, RecO plays an essential role in genetic recombination in the absence of *B. subtilis* RecA. RecO is alone required for plasmid establishment in natural competent cells, but RecR and RecF proteins are required for the repair of abortive transformation reaction when other pathways are blocked (e.g., *recF addAB* cells) (Makharashvili, Koroleva et al. 2004, Leiros, Timmins et al. 2005). This is consistent with: *i*) *in vivo* fluorescence microscopy data showing that upon addition of plasmid DNA, RecO accumulates at the cell pole in natural competent cells and *ii*) RecO promotes the annealing of complementary ssDNA molecules even in the presence of SsbA, in contrast to RecA which fails to promote strand annealing of SsbA covered complementary DNA (Carrasco, Manfredi et al. 2008). It has been described that other mediator proteins (e.g. bacterial RecFOR or eukaryotic BRCA2) can load the recombinase at gaps on the template DNA coated with a SSB protein or onto the

generated naked ssDNA (e.g. bacterial RecBCD) during DNA end processing (Spies and Kowalczykowski 2005).

The RecO protein of *B. subtilis* is a 225 -amino-acid polypeptide (Mm 29,195 Da, pI 8.21). The *recO* gene has a conserved genomic organization when compared with the $recO_{Eco}$ gene, but has poor identity at the protein level. Only the N-terminal region of RecO shares a low level of identity with $RecO_{Eco}$ (29% in the first 164 amino acids of the 255-residue long polypeptide), but the identity reduced significantly towards the C-terminal region of protein (Leiros, Timmins et al. 2005).

The Crystal structure of *B. subtilis* RecO is not known show far, however it have been crystallized and studied in some other bacteria. The crystal structure of $RecO_{Dra}$ has revealed that the N-terminal domain (NTD) adopts an oligonucleotide/oligosacharide-binding fold (OB-fold), which is also present in eukaryotic RPA or BRCA2 protein (Figure 5).

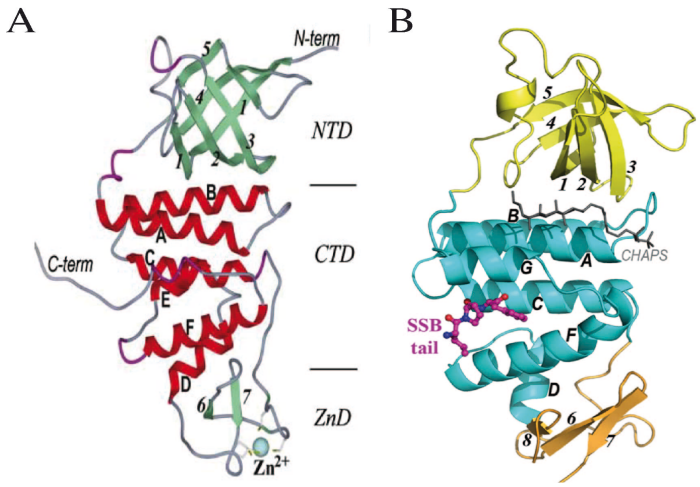


Figure 5: Crystal structure of RecO protein from *D. radiodurans*. (A) Crystal structure of RecO (B) co-crystal of RecO bound with SSB-ctail.

The C-terminal α-helical domain (CTD) composed of six α helices; and a zinc-binding domain (ZnD), which is inserted between αC and αD of the CTD (Makharashvili, Koroleva et al. 2004). The Zn^{2+} domain (ZnD) is present in RecO, Deinococcus or Mycobacterial RecO, but absent in $RecO_{Eco}$.

Co-crystal structure of the SSB_{Eco}-Ct, bound with $RecO_{Eco}$ reveal that SSB_{Eco}-Ct binds in the hydrophobic pocket of $RecO_{Eco}$. These hydrophobic interactions facilitate binding of SSB-Ct to $RecO_{Eco}$ and $RecO_{Eco}$/$RecR_{Eco}$ complex in both low and moderate ionic strength solutions. SSB_{Eco} binds $RecO_{Eco}$ to recrut $RecA_{Eco}$ onto ssDNA through SSB-Ct, and this interaction leads to modify RMPs conformation. Intriguingly, $RecO_{Dra}$ does not bind to SSB-Ct and weakly interacts with the peptide in the presence of $RecR_{Dra}$, suggesting the diverse mechanisms of DNA repair pathways mediated by RecO in different organisms (Inoue, Nagae et al. 2011, Ryzhikov, Koroleva et al. 2011). During recombinational repair, SsbA physically interacts with RecO as well as with many other proteins (Lecointe, Serena et al. 2007, Manfredi, Carrasco et al. 2008). This RecO-SsbA interaction is important for the cell growth, suggested by the observation of SsbA variant lacking the last C-terminal 35 residues (SsbA Δ 35), which have thermo sensitivity of *B. subtilis* cells upon expression of RecO (Costes, Lecointe et al. 2010).

1.4.4. DprA/Smf:

The DprA orthologs have been shown to convey an important role in transformation with chromosomal DNA or with plasmid DNA (Berka, Hahn et al. 2002, Ogura, Yamaguchi et al. 2002, Bergé, Mortier-Barriere et al. 2003). $DprA_{Spn}$ is thought to protect incoming DNA during transformation, but genetic and biochemical functions are poorly understood. $DprA_{Spn}$ protein is dependent on ComK for over-expression and it is competence-specific protein. DprA was first identified in *H. pylori* and shown to co-localize with ComGA at the cell pole, independent of RecA or RecN, but localizes throughout in cells lacking polar ComGA in *H. influenzae* (Berka, Hahn et al. 2002, Bergé, Mortier-Barriere et al. 2003, Quevillon-Cheruel, Campo et al. 2012).

DprA is a 297-amino-acid polypeptide (Mm 32783 Da, pI of 9.76). DprA shares 44 % identity with $DprA_{Spn}$. The crystal structure of $DprA_{Spn}$ shows that the protein form tail-to-tail dimers, and $DprA_{Spn}$ consists of two domains (Figure 6). The N-terminal domain is composed of five helices, and presents considerable structural similarity to the SAM domain of PA4738, a protein of unknown function from *P. aeruginosa*. SAM domains frequently are involved in various types of protein interactions (Figure 6A). The C-terminal region of $DprA_{Spn}$ adopts an RF-like topology with a typical three layers (αβα) sandwich (Figure 6B). The short region connecting the SAM and the RF is composed of two anti parallel β-strands, each followed by α-helices. Overall $DprA_{Spn}$ structure forms a bean shaped, globular structure (Quevillon-Cheruel, Campo et al. 2012).

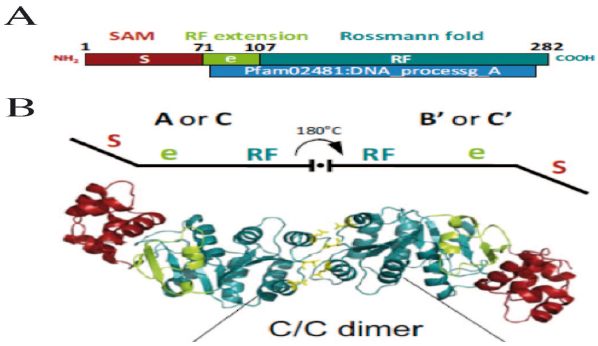


Figure 6: **Crystal structure of DprA from *S. pneumoniae***

It has been shown that $DprA_{Spn}$, which crystallizes as a dimer (Figure 6B), physically interacts with $RecA_{Spn}$ (Quevillon-Cheruel, Campo et al. 2012). DprA interacts with SsbB, RecA and with itself, shown by *in vivo* analyses (Kramer, Hahn et al. 2007). The $DprA_{Spn}$ binds with ssDNA, and facilitate the recruitment of the heterologous $RecA_{Eco}$ onto ssDNA in absence or presence of heterologous SSB_{Eco} on ssDNA. $DprA_{Spn}$ forms mix $DprA_{Spn}$·ssDNA·$RecA_{Eco}$ nucleoprotein filament. It was proposed that this mix complex is proficient for DNA strand exchange (Mortier-Barriere, Velten et al. 2007). It was proposed that $DprA_{Spn}$ might be the first protein to interact with naked ssDNA as soon as it exits from the entry channel. This interaction leads to formation of $DprA_{Spn}$·ssDNA complex, which might facilitate $RecA_{Spn}$ loading onto ssDNA for the genetic transformation process (Mortier-Barriere, Velten et al. 2007). The role of DprA as mediator is less understood in *B. subtilis* competent cells.

A possible role of $DprA_{Spn}$ has been suggested for plasmid transformation because it could anneal complementary ssDNA segments (Mortier-Barriere, Velten et al. 2007). DprA *($DprA_{Spn}$)* interacts physically with RecA ($RecA_{Spn}$) (Kramer, Hahn et al. 2007, Mortier-Barriere, Velten et al. 2007). In the case of *B. subtilis* and *H. pylori* competent cells, *in vivo* analyses reveals that RecA forms a focus (nucleation onto ssDNA?), and dynamics threads (RecA·ssDNA filament?) even in the absence of DprA (Kidane and Graumann 2005, Tadesse and Graumann 2007, Orillard, Radicella et al. 2011), which suggesting that another mediator

(*e.g.*, RecO) alone or in concert with a guardian protein (*e.g.*, SsbA, SsbB, RecN) might be also involved in RecA recruitment onto the internalized ssDNA. The DprA protein have been studied in different bacteria's but role of DprA from *B. subtilis* is remain unclear and need to understand for chromosomal and plasmid transformation.

1.4.5. CoiA/YjbF:

The function of CoiA/YjbF (a 373-residue polypeptide chain, Mm 43531 Da, pI 9.97) is not known. This protein is localized at the pole of competent cell, and most likely stabilized by interactions with one or more Com proteins (Kramer, Hahn et al. 2007). The absence of *coiA* has very minor effect on genetic recombination in both *S. pneumoniae* and *B. subtilis*. The $CoiA_{Spn}$ seems to function at a later stage than the guardian proteins (SSBs), and it was suggested to be involved in promoting recombination during genetic transformation (Desai and Morrison 2007). The biochemical activities associated with CoiA are unknown.

1.4.6. RecX, RecF:

The RecX (798 polypeptide, Mm 88,993 Da, pI 5.08) is a widespread bacterial protein. Localization and activation varies throughout bacterial system. In some cases the *recX* gene is present just downstream *recA* and express through the same promoter but expression of *recX* gene is limited (5 to 10% of *recA* level) because of presence of hairpin interruption between *recA* and *recX* genes. In other bacteria *recX* localizes away from *recA* gene, and its expression is not regulated by LexA (Drees, Chitteni-Pattu et al. 2006).

RecX is a negative modulator of RecA, and it inhibits RecA protein ATPase activity *in vitro*, its co-protease activity *in vivo*, and recombinase activity both *in vivo* and *in vitro*. In *E. coli* RecA and RecX proteins physically interact with each other, shown by yeast two-hybrid analyses (Stohl, Brockman et al. 2003). RecX interacts with the C-terminus of RecA (Drees, Chitteni-Pattu et al. 2006) or RecX can bind within the helical groove of the RecA nucleoprotein filament. $RecX_{Eco}$ acts primarily by blocking the growing end of $RecA_{Eco}$ filaments by capping mechanism (Drees, Lusetti et al. 2004). The inhibition of $RecA_{Eco}$ ATPase activity by $RecX_{Eco}$ is greater in the presence of SSB_{Eco} than in its absence (Baitin, Gruenig et al. 2008). RecX possesses a limited ssDNA binding activity (Stohl, Brockman et al. 2003, Drees, Lusetti et al. 2004).

Cytological analyses of the DSB repair response suggest that RecF might work after RecA nucleation (Kidane, Sanchez et al. 2004). RecF protein (370 polypeptide, Mm 42,146 Da, pI 7.01) physically interacts with the RecR protein in a manner dependent on both DNA and ATP. RecR protein has mainly been studied jointly with the RecF protein or with its other interacting partner, RecO. *In vivo*, in at least in some contexts, RecF functions on its own. RecF may have multiple functions, some of which may be independent of the RecO and RecR proteins (Lusetti, Hobbs et al. 2006). $RecF_{Eco}$ interacts physically with $RecX_{Eco}$ and destabilizes it from $RecA_{Eco}$ filaments. In *B. subtilis* RecX and RecF might modulate dynamic RecA·ssDNA filament extension (Paula P. Cárdenas 2012). In some bacterial species, RecA mediation and modulation, is catalyzed by the same protein (*e.g.*, $DprA_{Spn}$) (Mortier-Barriere, Velten et al. 2007), whereas in other species both activities might be carried out by different proteins (*e.g.*, RecO and RecX). It is proposed that RecX and RecF facilitates DNA repair and genetic recombination by modulating the "length or packing" of a RecA filament (Paula P. Cárdenas 2012)

1.5. Effect of nucleotide cofactor on genetic recombination:

B. subtilis is the best-studied member of the Firmicutes phylum. Since the evolutionary distance between *B. subtilis* and *E. coli* cells is greater than the one between plants and animals, this raises the question whether the regulation of RecA nucleation proceeds similarly in these two bacteria. $RecA_{Eco}$ is an ssDNA-dependent ATPase, but also has been found to efficiently hydrolyze dATP. rATP (termed here simply ATP) is hydrolyzed by $RecA_{Eco}$ resulted in ADP + Pi. This hydrolyzed ADP acts as a competitive inhibitor of $RecA_{Eco}$ for ATP hydrolysis, like ATPγS that cannot be hydrolyzed by $RecA_{Eco}$. The $RecA_{Eco}$ binding with ATP or ADP leads to change in different conformations of RecA filament on ssDNA (Lee and Cox 1990). If RecA filament formation is performed in the presence of dATP, the hydrolysis activity increases to 20% and strand exchange increases too, compared with that observed in the presence of ATP (Menetski, Varghese et al. 1988, Menetski and Kowalczykowski 1989). Several nucleotides bind to and are hydrolyzed by $RecA_{Eco}$ protein. These nucleotides influence the affinity of $RecA_{Eco}$ protein for ssDNA and induce a high-affinity ssDNA binding state of $RecA_{Eco}$ protein, which has a greater stability than that induced by any other nucleoside triphosphate. The dATP has a marked effect on many properties of $RecA_{Eco}$ protein. Perhaps most significantly, the dATP-dependent DNA strand exchange reaction is enhanced under conditions, which are not optimal for DNA strand exchange in the presence of ATP. These enhanced properties of the dATP-dependent DNA strand exchange reaction are presumably related to the increased ability of the dATP-$recA_{Eco}$ protein complex to compete for ssDNA binding sites. In the absence of SSB protein, the dATP-$RecA_{Eco}$ protein complex can disrupt more secondary structure in ssDNA than the ATP complex. While in the presence of SSB_{Eco}, dATP-$RecA_{Eco}$ can displace SSB_{Eco} protein from ssDNA more effectively than the ATP complex (Menetski, Varghese et al. 1988, Menetski and Kowalczykowski 1989).

$RecA_{Eco}$ forms nucleoprotein filaments preferentially on single-stranded (ss) DNA in the presence of ATP or a non-hydrolysable ATP analogue (ATPγS or AMP-PNP) with a stoichiometry of one $RecA_{Eco}$ monomer per 3 nucleotides (nt) (~18-nt per filament turn). SSB_{Eco} pre-bound to ssDNA limits $RecA_{Eco}$ nucleoprotein filament formation, and thereby repressing unwanted recombinase filamentation in the presence of dATP, ATP or a non-hydrolysable ATP analogue (ATPγS or AMP-PNP) (Kowalczykowski, Dixon et al. 1994, Bianco, Tracy et al. 1998, Cox 2007, Galletto and Kowalczykowski 2007).

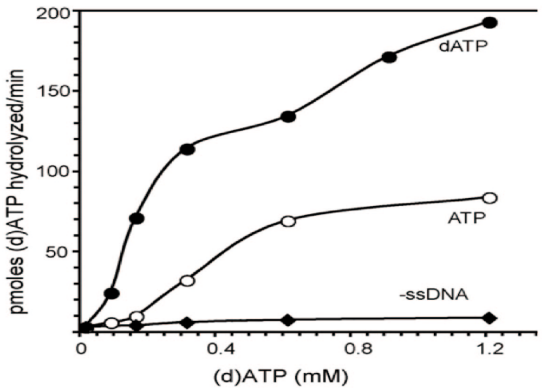


Figure 7: Effect of nucleotide cofactor on RecA activity:

Unlike $RecA_{Eco}$, RecA of Firmicutes origin shows a strong preference for dATP as a nucleotide cofactor *in vitro* (Figure 7) (Lovett and Roberts 1985, Steffen and Bryant 1999, Steffen, Katz et al. 2002, Carrasco, Manfredi et al. 2008, Grove, Anne et al. 2012). ATP is 100- to 500-fold more abundant than dATP *in vivo* (Mathews 1972, Bennett, Kimball et al. 2009), but RecA (or $RecA_{Spn}$) self-assembly onto cognate SSB proteins covering the ssDNA preferentially uses dATP as a nucleotide cofactor *in vitro* (Lovett and Roberts 1985, Steffen

and Bryant 1999, Steffen, Katz et al. 2002, Carrasco, Manfredi et al. 2008, Grove, Anne et al. 2012). It is unknown whether Firmicutes RecA is optimized for dATP utilization *in vivo*. At least two mechanisms for the inability of RecA (or $RecA_{Spn}$) to polymerize onto SSB-coated ssDNA in the presence of dATP can be envisioned. In the presence of dATP, RecA, $RecA_{Spn}$ or $RecA_{Eco}$ bind ssDNA more tightly and invade more secondary structure in ssDNA (Menetski and Kowalczykowski 1989) or can prevent a net end-dependent RecA disassembly (Shan and Cox 1997), but RecA in the presence of ATP, or even ATPγS, cannot displace SsbA from ssDNA (Carrasco, Manfredi et al. 2008, Chiesa, Cardenas et al. 2012). Alternatively, in the presence of ATP Firmicutes SsbA (or SsbB) displaces RecA from ssDNA (Steffen, Katz et al. 2002), suggesting that ATP bound RecA has to go through different conformational changes to overcome the SSB displacement from the ssDNA. In the absence of SsbA, RecA hydrolyzes dATP in preference to ATP at any Mg^{2+} ion concentrations tested, and competes slightly better for ssDNA binding with SsbA at Mg^{2+} concentrations between 4–10 mM. In the presence of ATP, at least 10 times more RecA is required to achieve a comparable level of strand exchange than in the presence of dATP (Carrasco, Manfredi et al. 2008).

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2. OBJECTIVES

2. Objectives:

1. Biochemical characterization of SsbA and SsbB from *B. subtilis*: nature of binding to ssDNA, and type of complexes, and their stability.

2. Role of SsbA and SsbB onto RecA nucleation and polymerization onto ssDNA, and their contribution to RecA-mediated DNA strand exchange.

3. Identification of genetic recombination mediators: involvement of RecO and DprA on chromosomal and plasmid transformation.

4. Role of RecO as RecA mediator: characterization of the potential SSB partner that works with RecO for the chromosomal transformation.

5. Biochemical characterization of RecO as single strand annealing protein.

6. Characterization of *B. subtilis* DprA for binding with DNA, possible interaction with SSB proteins.

7. Role of DprA as RecA mediator in presence of SSBs for the chromosomal transformation.

8. Biochemical characterization of DprA activity of complementary ssDNAs annealing ability, in the presence of SSBs, for plasmid transformation.

9. The effect of dATP or ATP on RecA nucleation and polymerization onto SsbA- and/or SsbB-coated ssDNA.

10. The effect of dATP, ATP or ATPγS on RecA-mediated three-strand exchange reaction.

3. MATERIAL AND METHODS

3. Materials and Methods

3.1. Materials

3.1.1. Strains

The strains used and / or constructed in this work are detailed in the following tables.

Table 1. *E. coli* strains

Strain	Genotype	Use
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10* (Tet)] (Stratagene)	Cells for construction and maintenance of plasmids.
BL21(DE3)	*pLysS B F-dcm ompT hsdS (rB-mB-) gal (DE3)* [pLysS Cat] (Stratagene)	Cell for overproduction of proteins: SsbA, SsbB, RecO, DprA etc

Table 2. *B. subtilis* strains

Strain	Relevant genotype[a]	Source or reference
BG214	*rec*$^+$	Lab. Collection
BG190	Δ*recA*	(Ceglowski, Luder et al. 1990)
BG439	Δ*recO*	(Fernandez, Kobayashi et al. 1999)
BG1163	Δ*dprA*	This study
BG1165	Δ*recO* Δ*dprA*	This study
BG649	Δ*recO* Δ*recA*	(Fernandez, Ayora et al. 2000)
BG1291	Δ*recA* Δ*dprA*	This study

3.1.2 media used

GM1: S-Base 1X, 0.5% glucose, 0.1% yeast extract, 0.02% hydrolyzed casein, 0.8 mM $MgSO_4$, 0.025%D / L-tryptophan, 0.02% L-methionine (Wilson and Bott 1968).

GM2: GM1 medium supplemented with 3.3 mM $MgSO_4$, and 0.5 mM $CaCl_2$ (Wilson and Bott 1968).

3.1.3. Buffers

Table 3: Buffers

Buffer	Composition
A	50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM DTT, 2 mM PMSF, 15 %glycerol.
B or C	50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol
D	50 mM KH_2PO_4/K_2HPO_4 pH 7.5, 1 mM DTT, 10% glycerol
E	50 mM KH_2PO_4/K_2HPO_4 pH 7.5, MgOAc 2mM, 5% glycerol
F	50 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM NaCl, 50 mg/ml BSA, 5% glycerol
G	50 mM Tris-HCl, pH 7.5, 1 mM DTT, 90 mM NaCl, 10 mM Mg(OAc), 50 μg/ml BSA, 5% glycerol
H	50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM EDTA, 110 mM NaCl, 50 μg/ml BSA, 5% glycerol
I	5 mM HEPES (pH 7.5), 5 mM EDTA, 65 mM NaCl, 5% glycerol

3. 2. Methods

3.2.1. Cell manipulation

3.2.1.1. Preparation of competent cells

Competent cells of *E. coli* were obtained as described: exponential *growing E. coli* cells were cultured in LB at 37° C with shaking until OD_{560} 0.4. Cells were harvested by centrifugation, and treated with 0.1 M $MgCl_2$ and 0.1 M $CaCl_2$, which permeabilizes cells by producing holes in the membrane, and they will be used to introduce DNA passively in the cells. The cells were stored at -80° C in the presence of 15% glycerol until use (Hanahan 1983).

B. subtilis competence develops naturally under certain growth conditions of nutrient limitation. To make *B. subtilis* competent cells a colony was inoculated in a liquid culture of GM1 which was incubated for 16 h at 30° C without agitation. This culture was used to inoculate fresh GM1 medium to OD_{560} 0.05 and incubated further at 37° C with vigorous agitation (250 rpm) until the cells reached the stationary growth phase. Cells were harvested, by centrifugation, after 90 min from point of stationary phase and stored at -80° C with 15% glycerol (Wilson and Bott 1968).

3.2.1.2. Chromosomal and plasmid transformation

E. coli transformation was carried out by following the heat shock method (Hanahan 1983). 200 µl competent cells were mixed with 10-100 ng of the plasmid and incubated at 4° C for 30 min. Then cells were heat shocked at 42° C for 1 min so the pores of the cell membrane were opened and the DNA was able to enter inside the cell. Cells were kept further on ice for 2 min, to restore the membrane. 1 ml of LB medium was added to the reaction, and incubated for 1 h at 37° C with stirring for cells to allow expression of plasmid-encoded antibiotic resistance gene(s). Then cells were plated on LB-agar plate, having selective antibiotics, and incubated 15 h at 37°C.

Cells of *B. subtilis* were transformed by the method of (Wilson and Bott 1968).Cells stored at -80° C were diluted in GM2 medium (1:10), and incubated for 1 to 3 h at 37°C under stirring. Then 200 µl of cells were mixed with 100-200 ng of DNA and incubated for 1 h at 37° C with shaking. Finally the cells were plated on LB-agar with the required antibiotic.

3.2.2. DNA manipulation

3.2.2.1. DNA purification and quantification

The cdsDNA plasmid was purified by alkaline lysis (Birnboim and Doly 1979) and subsequent CsCl gradient (Sambrook 1989) or by using the DNA purification kit from Qiagen. The cssDNAs of pGEM-3Z f (+) was obtained by culturing the *E. coli* cells carrying replicating plasmid and by infecting them with helper phage infective particles. The cells were removed by centrifugation, and the phage supernatant was precipitated with 20% PEG 6000 and 2.6 M NaCl. The ssDNA was obtained. Phenol extractions have been performed to remove proteins from phage, and subsequent ethanol precipitation for pure cssDNAs.

Chromosomal DNA from *B. subtilis* was extracted by common procedures (Sambrook 1989).

DNA concentration was quantified by absorbance at 260 nm and its purity was determined using a coefficient relating the absorbance at 260 nm and 280 nm of 2 (Sambrook 1989)

3.2.2.2. Plasmids

The plasmids used and / or constructed along the development of this work.

Table 4. Plasmids

Plasmid	Derived from	Descriptions and reference
pT712	pMini-ColE1	It has the T7 promoter of phage ϕ10 (GIBCO-BRL)
pET3a, pET21a*	pColE1	Has T7 promoter of phage ϕ10 (Novagen). *6 histidine tails introduced in the carboxyl terminal sequence of the cloned DNA.
pGEM 3Zf (+/-)	pMini-ColE1	It contains the origin of replication of phage ϕ1 in different orientation (-) and (+) (Promega).
pCB669	pT712	Contains the gene for RecO overexpression
pCB722	pET3a	Contains the gene for SsbA overexpression (Carrasco, Manfredi et al. 2008).
pUB110	-	Natural occurring plasmid.
pBT61	pUB110	Contains the *recA* gene of *B. subtilis* for overexpression (Gassel and Alonso 1989)
pCB777	pET3a	Contains the gene for SsbB overexpression (this work)
pCB892	pET3a	Contains the gene for SsbB* overexpression (this work)
pCB596	pET3a	Contains the gene for ssb_{SPP1} overexpression.
pCB888	pET21d	*dprA* gene under the control of a phage T7 promoter was used to over-express DprA in *E. coli* BL21(DE3)[pLysS] cells (this work).

3.2.2.3. DNA radiolabelling

The 440-nt fragment was obtained by PCR amplification using plasmid DNA pGEM3zf (+). PCR was performed with oligonucleotides, which hybridized at the positions of restriction sites EcoRI (position 5) and AflIII (position 445) and the fragment was gel purified from agarose gels. Subsequently proceeded to the marking of cDNAs in the 5' DNA end by incorporating [γ-^{32}P] with a treatment with T4 polynucleotide kinase (PNK), in the presence of 200 μM [γ^{32}P]-ATP, for 1 h at 37° C. The excess of non-incorporated nucleotides were removed by filtration, passing the reaction mixture by a Sephadex G-50, according to Sambrook (Sambrook 1989)

3.2.3. Protein purification

3.2.3.1. Over-expression and purification of proteins

RecA is over-expressed in growing *B. subtilis* BG214 cells bearing pBT61 (plasmid-borne recA gene) under its own promoter (Gassel and Alonso 1989), to OD_{560}~ 0.6. RecA expression was induced by addition of mitomycin C (MMC) to a final concentration of 250 ng/ml. Culture was allowed to grow for 2 h at 37° C with shaking. Then cells were centrifuged, and wet mass of cells frozen at -20° C until further use. RecA was purified as previously described (Carrasco, Ayora et al. 2005).

E. coli BL21(DE3) (pLysS) cells bearing pCB669-borne *recO* gene were grown to saturation with spontaneous auto-induction of the 29.3-kDa RecO protein, harvested, and resuspended in buffer A containing 300mM NaCl. The cells were disrupted by the addition of

lysozyme (500 ng/ml), followed by sonication. After centrifugation RecO was found in the soluble fraction. PEI was added to a final concentration of 0.25% (A_{260} = 120) and the mixture was spun at 30,000 *g* for 30 min. The PEI supernatant containing RecO was subjected to ammonium sulfate (AS) precipitation (30% saturation). The pellet was resuspended in buffer A to a final concentration of 30 mM NaCl, and loaded onto a Q-Sepharose column equilibrated with the same buffer. The flow-trough was loaded onto an S-Sepharose column. RecO was eluted by a step gradient from 60 to 200 mM NaCl. Fractions containing RecO (which was free of $RecO_{Eco}$, $RecR_{Eco}$, or $RecF_{Eco}$ protein) were pooled, concentrated, and stored in buffer A containing 300 mM NaCl and 50% glycerol at -20° C.

E. coli BL21(DE3) (pLysS) cells bearing the pCB722-borne *ssbA* gene were grown to mid-exponential phase and the expression of the 18.7-kDa SsbA protein was induced by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), and the cells were collected 90 min later. The cell mass was resuspended in buffer B (Table 3) containing 100 mM NaCl and disrupted by the addition of lysozyme (500 ng/ml), followed by sonication. After centrifugation, SsbA was found in the soluble fraction. PEI was added to a final concentration of 0.25% (A_{260} = 120) and the mixture spun at 30,000 *g* for 30 min. The SsbA protein was resuspended from the PEI pellet in buffer B containing 400mM NaCl and subjected to AS precipitation (30% saturation). The pellet was resuspended in buffer B containing 100 mM NaCl and loaded onto a Q-Sepharose column equilibrated with the same buffer. SsbA was eluted with a linear gradient from 150 to 400 mM NaCl. Fractions containing SsbA were recovered and loaded onto a hydroxyapatite column equilibrated with the same buffer. The column was washed with buffer B containing 10 mM KH_2PO_4/K_2HPO_4 and eluted with a 10–50 mM KH_2PO_4/K_2HPO_4 gradient. The peak fractions containing SsbA (which was free of SSB_{Eco}) were pooled, concentrated, and stored in buffer B containing 300 mM NaCl and 50% glycerol at -20° C.

E. coli BL21(DE3)[pLysS] cells bearing the pCB777-borne *ssbB* or pCB892-borne *ssbB** gene were grown at 18° to middle exponential phase and the expression of the 12.4 kDa SsbB or13.5 kDa SsbB* protein was induced by adding 0.3 mM IPTG. Cells grew an additional 15 h and were pelleted. The cell mass was resuspended in buffer C (Table 3) containing 1 mM PMSF and 1 M NaCl and lysed by sonication. After centrifugation SsbB or SsbB* was found in the soluble fraction. PEI was added to a final concentration of 0.25% (A260 = 120) and the mixture spun at 30,000 *g* for 15 min. The SsbB or SsbB* was collected from the PEI supernatant and subjected to AS precipitation (50% saturation). The pellet was resuspended in buffer C and loaded onto a Phenyl-Sepharose column equilibrated with buffer C containing 1 M AS. SsbB or SsbB* was eluted with a linear gradient from 1 to 0.1 M AS in buffer C. Fractions containing SsbB or SsbB* were recovered and loaded onto a Butyl-Sepharose column equilibrated buffer C containing 0.5 M AS. SsbB or SsbB* was eluted by linear gradient from 0.5 M to 0.1 M AS in buffer C. Fractions containing SsbB or SsbB* were recovered and loaded onto a hydroxyapatite column equilibrated with buffer C containing 0.2 M AS. The column was washed with buffer C containing 5 mM KH2PO4/K2HPO4 pH 7.5 and 0.2 M AS, and eluted with a linear gradient from 5 mM - 50 mM KH2PO4/K2HPO4 gradient. The fractions containing SsbB or SsbB*, which were free of SSB_{Eco}, were pooled, concentrated, and stored in buffer C and 50% glycerol at -20° C.

E. coli BL21(DE3)[pLysS] cell bearing the pCB888-borne *dprA* gene was grown at 25° C to middle exponential phase and the expression of the 32.7 kDa DprA protein was induced by adding 0.4 mM IPTG. Rifampicin (200 μg/ml) was added in culture after 30 min of IPTG induction. Cells grew an additional 3 h and were pelleted. The cell mass was resuspended in buffer D (Table 3) containing 1 mM PMSF and 1 M NaCl and lysed by sonication. After centrifugation DprA was found in the soluble fraction and loaded onto a Ni^{2+}-chelating column equilibrated with buffer D containing 1 M NaCl and 5 mM Imidazol. DprA was eluted with a linear gradient of Imidazol from 10 mM to 200 mM, in buffer D with 1 M NaCl. Fractions containing DprA were recovered and dialyzed against buffer D containing 400 mM NaCl. Dialyzed DprA was loaded onto a SP-sepharose column with Buffer D containing 150 mM NaCl. DprA was eluted with a linear gradient of NaCl from 200 mM to 1 M, in buffer D.

Fractions containing DprA were recovered and dialyzed against buffer D containing 300 mM NaCl, and stored in buffer D and 50% glycerol at -20° C.

3.2.4. Characterization / analysis of Protein

All proteins were purified to homogeneity greater than 98%. The NH_2 terminus of the purified proteins was sequenced by automatic Edman degradation. The corresponding molar extinction coefficients for SsbA, SsbB, SsbB*, RecA and RecO were calculated as 11,400, 13,000, 12,950, 15,200 and 19,600 M^{-1} cm^{-1}, respectively, at 280 nm, as previously described (Carrasco, Ayora et al. 2005). The protein concentrations were determined using the above molar extinction coefficients, and RecA is expressed as mol of protein as monomers, RecO as dimers, and SsbA, SsbB and SsbB* as tetramers.

The purified proteins were also identified, by the CNB Proteomics Service, by trypsin digestion method (MALDI-MS).

3.2.5. Biochemical Assays

3.2.5.1. Determination of the oligomeric state of SsbA and SSbB:

For determination of the oligomeric state of SsbA or SsbB protein cross-linking experiments were performed. A constant amount of SsbA or SsbB was incubated in the presence or absence of the cross-linking agent glutaraldehyde (0.05%) for 15 min at 37º C in buffer E (Table 3) containing 50 mM NaCl, in a 20µl reaction. The proteins were separated in a gradient from 10% to 15% PAGE. Both SsbA and SsbB were tetramers.

3.2.5.2. Protein and DNA interactions

The formation of SsbA-, SsbB- or SsbB*-ssDNA complexes were measured by EMSA or filter binding assays.

A: Electrophoretic mobility shift assay (EMSA)

ssDNA segment of different length (30-, 40-, 50-, 60-, 70- and 80-nt long poly [dT] or natural occurring ssDNA were end-labelled with [γ-^{32}P] (0.2 nM in ssDNA molecules). The labelled ssDNA segmentswas incubated with various amounts of SsbA or SsbB proteins for 15 min at 37° C in buffer F (Table 3) containing or not 10 mM magnesium acetate (MgOAc) in a final volume of 20 µl. The mixture was stopped and separated either using a 10% PAGE. The PAGEs were run with Tris-borate at 45 V at 4° C and dried prior to autoradiography.

B: Filter binding assay

The rate of dissociation of the SsbA- or SsbB-ssDNA complexes was measured by using alkali-treated filters (millipore, type HAWP 0.45 µm) as previously described (Riggs, Bourgeois et al. 1970, Alonso, Stiege et al. 1993). The 40-, 50-, 60-, 70- or 80-nt long [γ-^{32}P]-poly[dT] ssDNA (0.2 nM in ssDNA molecules) was pre-incubated with a fix SsbA or SsbB concentration for 15 min at 37° C in buffer F. Then 20-fold excess of cold poly[dT] ssDNA was added (time zero) and sampling begun. The reaction mixture was stopped by 1 ml ice-cold buffer F and then filtered trough KOH-treated filters. Filters were dried and the amount of radiactivity bound to the filter was determined by scintillation counting. The DNA retained on filter was corrected for using the retention of radiolabeled DNA in the absence of protein. The specific activity of the labelled DNA was measured as 10% TCA precipitable material. All reactions were performed in duplicate.

3.2.5.3. RecA (d)ATP hydrolysis assay

The ssDNA-dependent dATP (or ATP) hydrolysis activity of RecA protein was observed via a coupled spectrophotometric enzyme assay (Morrical, Lee et al. 1986, Hobbs, Sakai et al. 2007). Absorbance measurements were taken with a Shimadzu CPS-240A dual-beam spectrophotometer equipped with a temperature controller and 6-position cell chamber. The cell path length and band pass were 1 cm and 2 nm, respectively. The regeneration of dATP (ATP) from dADP (ADP) and phosphoenolpyruvate driven by the oxidation of NADH can be followed by a decrease in absorbance at 340 nm. Rates of ssDNA-dependent RecA-mediated dATP (ATP) hydrolysis and the lag times were measured in buffer G (Table 3) containing 5 mM dATP for variable time at 37° C in a 100 µl reaction mixture. A dATP (ATP) regeneration system (0.5 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase) and a coupling system (0.25 mM NADH, 10 units/ml lactate dehydrogenase, 3 mM potassium glutamate) were also included. The orders of addition of 3,199-nt pGEM ssDNA (10 mM in nt), the proteins and their concentrations were indicated in the text. The amount of dADP (ADP) accumulated was calculated as describe (Arenson, Tsodikov et al. 1999).

3.2.5.4. RecA-mediated dATP-dependent DNA strand exchange

The 3,199-bp *Kpn*I-cleaved dsDNA (20 µM in nt) and homologous circular 3,199-nt ssDNA (10 µM in nt) were incubated with the indicated concentrations of the indicated protein or protein combination in buffer G containing 2 mM dATP (ATP) for variable periods or for 60 min at 37° C in a final volume of 20 µl. A dATP (ATP) regeneration system was included when indicated. The samples were deproteinized as described (Ayora, Missich et al. 2002, Ayora, Weise et al. 2002), and fractionated through 0.8% agarose gel electrophoresis (AGE) with ethidium bromide. The signal was quantified using a Geldoc (BioRad) system as described (Carrasco, Manfredi et al. 2008).

3.2.5.5. Complementary DNA strand annealing

Linear 440-bp [γ-^{32}P]-dsDNA was heat denatured during 10 min at 100º C and shifted to water-ice for 2 min. Heat-denatured linear 440-nt [γ-^{32}P]-ssDNA (7 mM in nt) was pre-incubated with SsbA, SsbB, SsbB* or both SsbA and SsbB (100 nM) for 10 min at 30º C in buffer H (Table 3) as described (Manfredi, Suzuki et al. 2010). Then variable amounts of RecO (or DprA) (0.1 to 3 µM) were added and reactions incubated for 60 min. The complexes formed were deproteinized as described (Ayora, Missich et al. 2002), and fractionated through 6% PAGE. The signal was quantified using a Geldoc (BioRad) system as described (Manfredi, Suzuki et al. 2010).

3.2.5.6. Determination of the C-ter exposure in SsbB*

Limiting Trypsin (0.25 µg/ml) was used to partially proteolyze free SsbB or SsbB* or ssDNA-bound SsbB or SsbB*, and the resulting products were separated using 20% SDS-PAGE. Tryptic digestion of gel-purified protein bands and their spotting onto the MALDI-targets (Voyager DE-STR, PerSeptive Biosystems, Foster City, USA) were performed as described (Lioy, Martin et al. 2006). The MALDI-TOF-TOF measurements of spotted peptide solutions were carried out on a Proteome-Analyzer 4700 (Applied Biosystems, Foster City, USA) as described previously (Soberon, Lioy et al. 2011).

3.2.5.7. Atomic force microscopy (AFM)

The formation of SsbA·, SsbB· or DprA·ssDNA complexes was measured by atomic force microscopy (AFM) in Buffer I (Table 3) containing 50 µM spermidine. The circular 3,199-nt ssDNA pGEM3 Zf(+) and Zf(-) (Watson and Crick strands) substrates were purified

as described in Supplemental material. pGEM3 Zf(+) ssDNA was incubated with the indicated protein for 10 min at 37° C in a 20 μl reaction mixture. A fraction of the sample was deposited on a freshly cleaved mica surface and the sample processed as previously described (Manfredi, Suzuki et al. 2010). AFM observations were performed on a Nanoscope IIIa (Digital Instruments) in air using the tapping mode. The cantilever (OMCL-AC160TS-W2, Olympus) was 129 mm in length with a spring constant of 33-62 N/m. The scanning frequency was 2-3 Hz, and images were captured with the height mode in a 512 X 512 pixel format. The obtained images were plane-fitted and flattened by the computer program accompanying the imaging module. The "*tip effect*" was removed using the apparent size of DNA as a reference. Volume analysis was carried out using the *Image SXM* software (Bickmore 1999). Image processing of the topographs and height measurements were performed as described (Pratto, Suzuki et al. 2009).

To calculate the theoretical volume of the particle employment equation (Schneider, Larmer et al. 1998):

$$Vt = (M_0/N_0)\ (V1 + d_*V2),\ \text{[equation 1]}$$

Where M_0 is the molecular weight, N_0 is the Avogadro's number; V1 and V2 are the partial specific volumes of water and protein, respectively (0.74 $cm^3\ g^{-1}$ and 1 $cm^3\ g^{-1}$, respectively), and d is the amplitude call hydration percentage of protein (0.4 mol H_2O/ mol protein).

To calculate the volume of experimental data measured height and width of the complex of the DNA termini was checked and depending on the height and width of the nearest dsDNA. Then, apply the following equation (Schneider, Larmer et al. 1998):

$$Vm = (h/6)\ (3_*r^2+h^2)\ \text{[Equation 2]}$$

Where Vm is the molecular volume, h and r are the height and radius the complex, respectively. Then, the data were represented in a frequency histogram (in nm^3) and analyzed using Gaussian curve fitting to obtain the mean value, using the Origin 6.0 software.

For DprA mediated strand annealing AFM analysis, the 3,199-bp pGEM3 Zf(+) ssDNA and its complementary pGEM3 Zf(-) ssDNA were incubated in Buffer I containing 50 μM spermidine with the indicated protein(s) for 30 min at 30° C in a 20 μl reaction mixture. A fraction of the sample was deposited on a freshly cleaved mica surface and the sample processed as previously described (Manfredi, Suzuki et al. 2010).

4. RESULTS

4. Results:

4.1. The SSB-like proteins from *B. subtilis*

B. subtilis encodes two SSB proteins. SsbA (counterpart of SSB_{Eco}) is a 172-residue long polypeptide that shares significant sequence identity with the DNA-binding N-terminal domain and protein-binding C-terminus of SSB_{Eco} or Ssb_{SPP1} (34-36%). SsbA is an essential homotetrameric protein involved in genome replication and maintenance. Unlike SsbA, $SsbA_{Spn}$ is not induced during competence development.

SsbB is a 113-residue polypeptide that is specialized for activity in transformational recombination, namely protection of internalized ssDNA. Homotetrameric SsbB shares 63% identity with the N-terminal DNA binding domain of SsbA (amino acids 1–106), but lacks the characteristic C-terminal tail that mediates protein interactions in SsbA. *In vivo* analyses in *B. subtilis* reveal that SsbB is located at the DNA entry poles in competent cells, and is in close content with RecA and DprA, whereas the localization of SsbA is unknown.

4.1.1. Biochemical characterization of SsbA and SsbB

The ssDNA, which is an intermediate in DNA replication, recombination and repair, is coated by SSB proteins. These proteins, which have been often considered as inert and protective of ssDNA, play a complex role in GR.

4.1.1.1. SsbA and SsbB ssDNA binding

SSB_{Eco} is a homotetramer that exhibits multiple binding modes differing in the number of monomers that interact with the ssDNA (Lohman and Ferrari 1994, Shereda, 2008). In general, under modest Mg^{2+} concentrations and low protein to ssDNA ratios, SSB_{Eco} uses all four subunits of the tetramer to bind ssDNA in the so called SSB_{65} binding mode, where 65-nt of ssDNA are occluded per SSB_{Eco} molecule. However, in the absence of Mg^{2+} and with higher protein to ssDNA ratios, SSB_{Eco} uses only two of its four subunits to interact with the ssDNA in the SSB_{35} binding mode (Lohman and Ferrari 1994, Shereda, 2008). To gain insight into the ssDNA binding and the type of nucleoprotein complexes formed by SsbA and SsbB

Proteins, binding assays with homopolymeric or heteropolymeric ssDNA were performed and the effects of Mg^{2+} were examined.

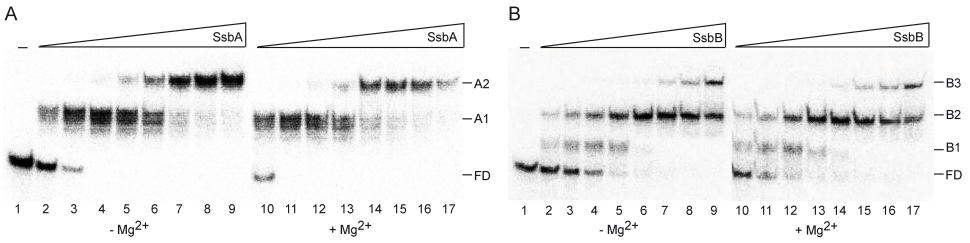

Figure 8: SsbA and SsbB binding with polydT80 in absence and presence of Mg^{2+}. (A and B), an 80-nt long [γ-^{32}P]-dT ssDNA (0.1nM in ssDNA molecules) was incubated with increasing concentrations of SsbA (0.03, 0.06, 0.12, 0.25, 0.5, 1, 2 and 4 nM) (A) or SsbB (0.8, 1.5, 3, 6, 12, 25, 50 and 100 nM) (B) in buffer F containing 5 mM EDTA (-Mg^{2+}) or 10 mM MgOAc (+Mg^{2+}) for 15 min at 37° C. The reactions were analyzed by 10% PAGE using a gel running buffer consisting of Tris-borate (pH 7.5) and the same concentration of MgOAc or EDTA as in the reaction solutions at 45 V at 4° C and dried prior to autoradiography. The bands corresponding to unbound poly(dT) (FD) and the various protein-ssDNA complexes (A1–A2 and B1–B3) were visualized by autoradiography.

SsbA bound homopolymeric dT_{80} in a concentration-dependent manner with an apparent dissociation constant (Kd_{app}) of <0.2nM in the absence or presence of Mg^{2+} (Figure 8

and Table 5). In low protein to dT_{80} ratios, an initial complex (A1) was formed with gel mobility lower than that of free dT_{80}, likely corresponding to ssDNA interacting with all four subunits of the tetramer (Figure 8 A, lanes 2–4 and 10–13). However, in the presence of higher protein to dT_{80} ratios (1 SsbA tetramer/ 32-nt or lower), the A1 product was no longer present and an A2 complex accumulated (Figure 8 A, lanes 5–9 and 14–17).

SsbB bound $polydT_{80}$ with a Kd_{app} of ~1.0 nM in the absence or presence of Mg^{2+}, a >5-fold lower affinity than SsbA (Figure 8B and Table 5). At low SsbB to $polydT_{80}$ ratios, two complexes were formed with gel mobilities lower than that of FD (B1 and B2; Figure 8B, lanes 2–5 and 10–13). Higher SsbB to $polydT_{80}$ ratios resulted in the disappearance of B1 and accumulation of B2, while saturating SsbB concentrations resulted in the appearance of a third, higher molecular-weight complex (B3; Figure 8 B, lanes 7–9 and 15–17). Unfortunately, nucleotide ratios could not be calculated due to indistinct formation of B1, B2 and B3. In general, a higher protein to $polydT_{80}$ ratio produced SsbB-ssDNA complexes with lower gel mobility. These data, along those of SsbA, support a model in which both SsbA and SsbB can bind $polydT_{80}$ in two binding modes, similar to those observed with SSB_{Eco}.

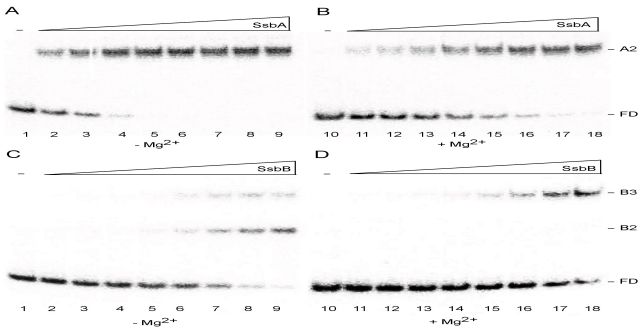

Figure 9: SsbA and SsbB binding with oligo in absence and presence of Mg2+. Binding of SsbA or SsbB to ssDNA. (A and **B)** an 80-nt long [γ^{-32P}]-ssDNA (0.1nM in ssDNA molecules) was incubated with increasing concentrations of SsbA (0.06, 0.12,0.25, 0.5, 1, 2, 4 and 8 nM) **(A** and **B)** or SsbB (0.4, 0.8, 1.5, 3, 6, 12, 25 and 50 nM) **(C** and **D)** in buffer F containing 5 mM EDTA (-Mg^{2+}, **A** and **C**) or 10 mM MgOAc (+Mg^{2+}, **B** and **D**) for 15 min at 37° C in a final volume of 20 µl. The reactions were analyzed as described in Figure 8.

SsbA and SsbB binding to a mixed-sequence ssDNA (heteropolymeric) of 80-nt in length with self-annealing potential was distinct from that observed with dT_{80}. SsbA bound this ssDNA with a Kd_{app} of <0.2nM and ~ 0.2nM in the absence and presence of Mg^{2+}, respectively, while SsbB bound with a Kd_{app} of 10 and 30 nM in the absence and presence of Mg^{2+} (Table 5). In terms of the number of complexes formed, SsbA binding of ssDNA led to the formation of the slow mobility complex (A2) (Figures 9A and 9B, lanes 2–9 and 11–18) regardless of Mg^{2+}. SsbB in the absence of Mg^{2+} formed both B2 and B3 complexes, while only B3 was observed in the presence of Mg^{2+} (Figures 9C and 9D, lanes 6–9 and 15–18). In both SsbA and SsbB interactions, a higher ratio of protein to heteropolymeric ssDNA was necessary for the complete binding of free ssDNA independent of the presence or absence of Mg^{2+} (Figures 9A to 9D). This is possibly the result of SsbA and SsbB binding ssDNA with secondary structure potential with lower affinity than homopolymeric ssDNA as shown by the Kd_{app}.

In contrast to the $polydT_{80}$ results where one or two SsbA tetramers appeared to bind $polydT_{80}$ in the presence or absence of Mg^{2+}, respectively, only one tetramer appeared to bind heteropolymeric ssDNA regard less of Mg^{2+} status. SsbB showed similar results but with dependence on Mg^{2+}.

To gain insight in the length of ssDNA needed for stable interactions with SsbA or SsbB, binding assays using dT_{20}, dT_{30}, dT_{40} and dT_{60} were performed in the presence or the absence of Mg^{2+}.

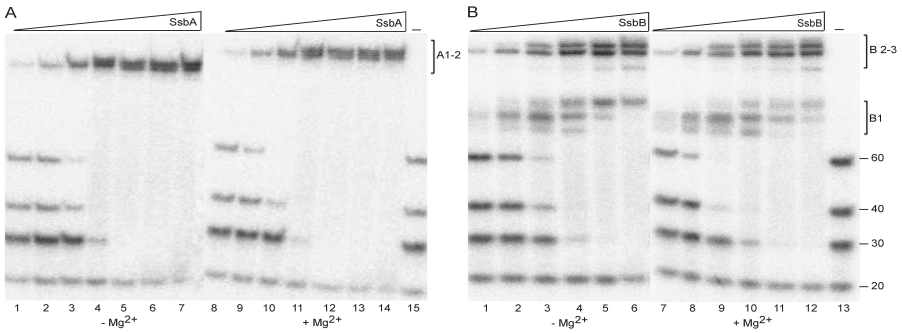

Figure 10: SsbA and SsbB binding with dT20/30/40/60 in absence and presence of Mg^{2+}. [γ-^{32P}]-poly (dT) ssDNA of different length (20-, 30-, 40- and 60-nt) (0.1nM in ssDNA molecules) was incubated with increasing concentrations of SsbA (0.015, 0.03, 0.06, 0.12, 0.25 and 1 nM) (C) or SsbB (3, 6, 12, 25, 50 and 100 nM) (D) in buffer F (-Mg^{2+} or +Mg^{2+}) for 15 min at 37° C. The reactions were analyzed as described in Figure 8.

Both proteins failed to bind polydT$_{20}$ but could bind the remaining ssDNAs regard less of Mg^{2+} status, (Figures 10A and 10B). In addition, both SsbA and SsbB appeared to have higher affinities for longer ssDNA segments (60> 40> 30), as lower protein concentrations were required to gel shift longer DNA (Figures 10 A and B). Paralleling the polydT$_{80}$ results, SsbA·polydTn complexes migrated as a single species regardless of SsbA concentration (A1–2), whereas the SsbB-polydTn complexes migrated as multiple species depending on the SsbB to ssDNA ratio (B1–2 and B3) (Figures 10C and 10D). Binding experiments done with individual dTn oligos confirmed these overall observations (data not shown).

Table 5: SsbA and SsbB binding quantification

DNA substrate	DNA binding affinity (in nM)			
	SsbA		SsbB	
	- Mg^{2+}	+ Mg^{2+}	- Mg^{2+}	+ Mg^{2+}
polydT$_{80}$ [a]	<0.2	<0.2	1.5 ± 0.5	1.2 ± 0.2
ssDNA$_{80}$ [a]	<0.2	0.20 ± 0.1	10 ± 5	30 ± 4
polydT$_{80}$ [b]	1.5 ± 0.5	ND	>200	ND

The Kd_{app} values (in nM) are the average of at least three independent experiments and are within a 10% standard error. [a]Proteins were incubated with the indicated substrate for 15 min at 37° C in buffer A containing or not 10 mM $MgCl_2$. Samples were separated by 10 % PAGE, and the formation of protein/DNA complexes was quantified as described in Materials and Methods. [b]Proteins were incubated with the indicated substrate for 15 min at 37° C in buffer A lacking $MgCl_2$. The mixture was filtered through KOH-treated filters (millipore, type HAWP 0.45 μm), the filters dried and the amount of radioactivity bound to the filter was determined by scintillation counting. ND, not done.

4.1.2. SsbA and SsbB ssDNA binding by Filter Binding Assay

To understand the origin of the above differences we measured the apparent thermodynamic stability (binding affinity) and kinetic stability (half-life) of the protein-ssDNA complexes by filter binding assays at low NaCl concentrations (~100 mM) in the absence of Mg^{2+}.

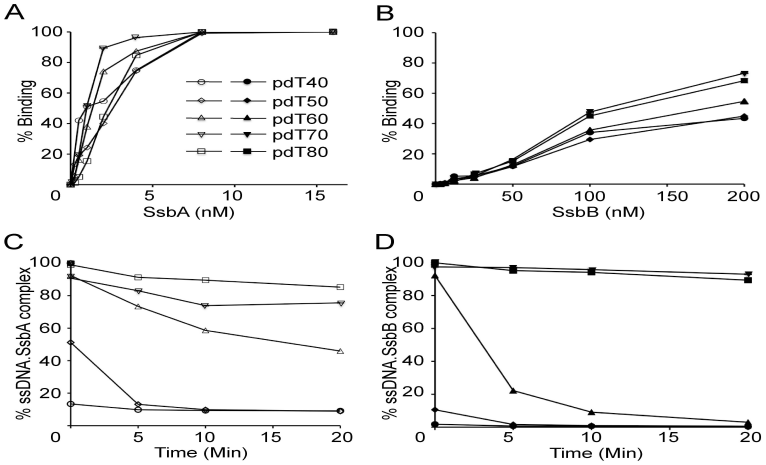

Figure 11: SsbA and SsbB bind homopolymeric dT80 ssDNA with different strengths. **(A** and **B),** an 80-nt long [γ-32P]-polydT ssDNA (0.2 nM in ssDNA molecules) was incubated with increasing concentrations of SsbA (0.25, 1, 2, 4, 8 and 16 nM) **(A)** or SsbB (3, 6, 12, 25, 50, 100 and 200 nM) **(B)** in buffer F containing 5 mM EDTA for 15 min at 37° C in a final volume of 20 µl. The reactions were filtered trough KOH-treated filters (Millipore, type HAWP 45 µm), the filters dried and the amount of radioactivity bound to the filter was determined by scintillation counting. **(C** and **D),** an 80-nt long [γ-32P]-polydT ssDNA (1 nM in ssDNA molecules) was incubated with increasing concentrations of SsbA (0.25, 1, 2, 4, 8 and 16 nM) **(C)** or SsbB (3, 6, 12, 25, 50, 100 and 200 nM) **(D)** in buffer F containing 5 mM EDTA for 15 min at 37°C in a final volume of 20 µl. Then 20-fold excess of cold polydT ssDNA in 80 µl was added (time zero) and 20 µl sampling begun. The reaction mixture was stopped by 1 ml ice-cold buffer F containing 5 mM EDTA and then filtered trough KOH-treated filters. Filters were dried and the amount of radioactivity bound to the filter was determined by scintillation counting. The DNA retained on filter was corrected for using the retention of radiolabeled DNA in the absence of SsbA or SsbB proteins. The specific activity of the labeled DNA was measured as 10% TCA precipitable material.

Both SsbA and SsbB form complexes with polydT80 with Kd_{app} ~1.5 and >200 nM, respectively (Figures 11A and 11B). The SsbA·ssDNA complexes were short lived when the ssDNA was 50-nt or shorter and the half-life increased significantly with polydT60 or longer oligos (Figure 11C). A similar pattern was observed for SsbB·ssDNA, except that SsbB also formed short-lived complexes with polydT60 ssDNA (Figure 11D). These data indicate that formation of SsbA·ssDNA and SsbB-ssDNA complexes was reduced ~7- and >200-fold, respectively, when comparing EMSA (Figures 8 A and B) and filter binding assays (Figures 11A and 11B). In general, SsbA appears to bind ssDNA with greater kinetic stability than SsbB in the absence of Mg^{2+}, corresponding to the homopolymeric and heteropolymeric results.

4.1.3. SsbA· and SsbB·ssDNA complex formation by AFM

To gain an insight into the mechanism by which ssDNA interacts with SsbA and/or SsbB, AFM experiments were performed (Figure 12). The protein·ssDNA binding experiments were performed in the absence of Mg^{2+} with the aim of detecting any possible difference between SsbA and SsbB proteins when bound to ssDNA.

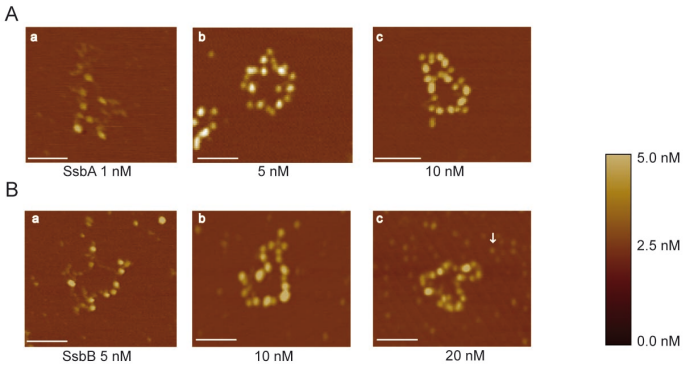

Figure 12: Binding of SsbA and SsbB to 3,199-nt pGEM3 Zf(+) ssDNA . (**A** and **B**), pGEM3 Zf(+) ssDNA (0.1 nM in ssDNA molecules) was incubated with increasing concentrations of SsbA (1, 5, or 10 nM) (**A**) or SsbB (5, 10, or 20 nM) **(B)** in Buffer I containing 50 μM spermidine for 10 min at 37° C.

The naked circular ssDNA (3,199-nt in length, pGEM3 Zf [+]) behaved as a disordered coil that made length measurements difficult. The mean height of the collapsed ssDNA was ~ 0.4 nm (data not shown) which deviated from the theoretical height for ssDNA (1 nm), confirming that ssDNA usually appears smaller than normal in AFM images (Vesenka, Guthold et al. 1992, Wyman, Grotkopp et al. 1995).

SsbA or SsbB specifically bound to ssDNA (Figures 12A and 12B), but failed to form a stable complex with duplex DNA (Manfredi, Suzuki et al. 2010, data not shown). At low ratios (1 SsbA/ 320-nt), SsbA extended the collapsed state of the partially naked ssDNA, and facilitated the formation of discrete beads of ssDNA·protein complexes, with an average as low as ~ 6 ± 2 SsbA beads per ssDNA molecule (Figure 12, n =200). At a ratio of 1 SsbA / 64-nt, circular beaded complexes were more densely packed, with an average of ~ 24 ± 4 SsbA beads per ssDNA molecule (Figure 12A). By contrast, at ratios of 1 SsbB/ 320-nt only naked ssDNA was observed (data not shown), consistent with the lower Kd_{app} of SsbB when compared to SsbA (see Introduction). At a ratio of 1 SsbB/ 64-nt, there were an average of ~ 10 ± 2 SsbB beads per ssDNA molecule (Figure 1B, n =150), and at a ratio of 1 SsbB/ 32-nt, this increased to ~ 24 ± 4 SsbB beads per ssDNA molecule (Figure 12B). The number of beads per ssDNA was 2 - 3-fold lower (i.e., 3199-nt/35-nt ~ 90 or 3199/65 ~ 50) smaller if the ssDNA lacked secondary structures.

The morphologies of the SsbA·ssDNA and SsbB·ssDNA complexes were similar in these experiments (Figures 12A and 12B), and were consistent with observed AFM images of tetrameric SSB_{Eco} bound to different circular ssDNA molecules in the presence or absence of Mg^{2+}, respectively (Hamon, Pastre et al. 2007, Li and Goh 2010). It is likely, therefore, that an SsbA or SsbB tetramer is the ssDNA binding unit. To test this hypothesis, the volume of the SsbA or SsbB beads was estimated. The observed volumes of SsbA (~ 135 ± 30 nm^3) and SsbB (~ 109 ± 22 nm^3) were in good agreement with: i) the theoretical volume of tetrameric SsbA (~ 120 nm^3) and SsbB (~ 82 nm^3), and ii) the volume determinations for SsbA, as well as volumes that were deduced from the co-crystal structures of SsbB·ssDNA, respectively (Manfredi, Suzuki et al. 2010, Yadav, Carrasco et al. 2012). At low ratios (1 SsbA or SsbB / 320- to 64-nt), the SsbA or SsbB beads had a height of ~ 1.6 ± 0.20 nm, and this height slightly increased to ~ 1.8 ± 0.18 nm at a higher protein·ssDNA ratio (1 SsbA/ 32-nt). The results, showing beaded morphology along the circular DNA, suggest that ssDNA wound around a SsbA or SsbB tetramer. At present we cannot rule out the possibility that at low SSB to ssDNA ratios both proteins may bind ssDNA in one binding mode, and that at high SSB to ssDNA ratios another binding mode may be favoured. For example, the SSB_{35} binding mode is favored at high SSB_{Eco} to ssDNA ratios (see Lohman, Overman et al. 1986).

To establish whether SsbA and SsbB bind ssDNA independently, or if there is a coordinate interaction between them, stoichiometric amounts of both SsbA and SsbB were co-assembled onto ssDNA (1 SSB/ 32-nt). Regardless of which protein was added first, there were ~ 24 ± 3 (SsbA added first) and ~ 27 ± 3 (SsbB added first) SSB beads per ssDNA

molecule (data not shown). It is likely that SsbA and SsbB interact preferentially with available ssDNA tracts that are free of partial duplex structures, such as hairpins.

4.1.4. SsbA and SsbB should bind sDNA in a similar fashion

The structure of full-length SsbB bound to polydT_{35} was determined at 2.8Å resolution X-ray crystal structure (Figure 13A). In the structure one SsbB tetramer bound to dT_{35} in a molar ratio of two dT_{35} oligos per SsbB (Figure 13) (Yadav et al 2012). The full SsbB tetramer comprised four monomers, or two symmetric SsbB pairs, with two protein monomers per asymmetric unit of the SsbB·dT_{35} complex (Figure 13) (Yadav et al. 2012) In total, 48-nt were fit to electron density, wrapping around the surface of the tetramer (24-nt in each crystallographic asymmetric unit) (Yadav et al 2012). Gaps between the observed polydT segments could be estimated to account for the remaining nucleotides, consistent with the apparent site size of ~60-nt for SsbB.

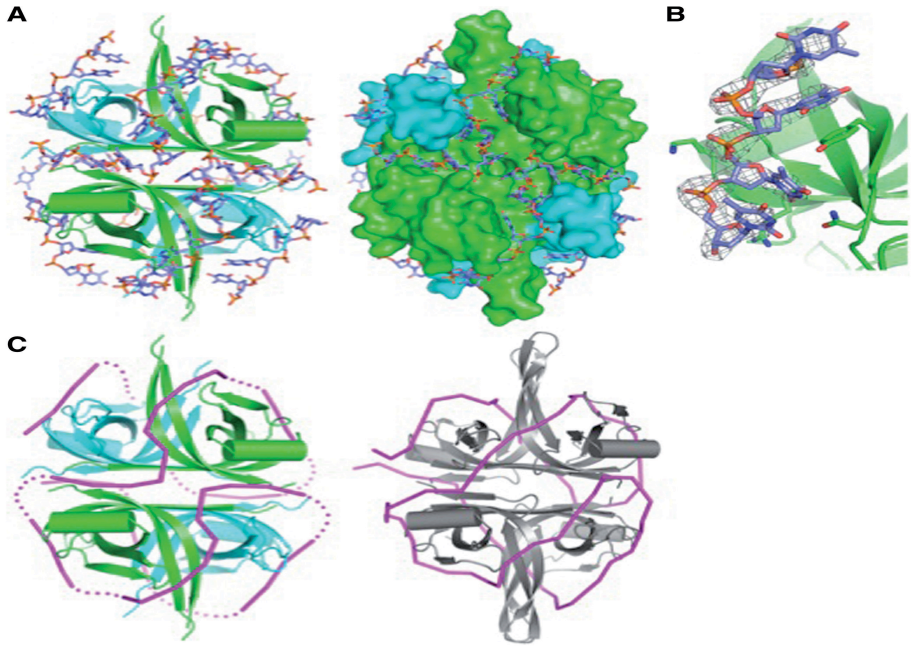

Figure 13. Structure of the SsbB·ssDNA complex (A) Structure of SsbB tetramer bound to dT35. Ribbon (left) and surface (right) diagrams show theSsbB tetramer (green and blue) with resolved dT35 (stick form). (B) 2Fo-Fc electron density contoured to 1.8 s showing an example of the dT_{35}bound to SsbB through stacking and electrostatic interactions. (C) Comparison of the SsbB·ssDNA (left) and SSBEco·ssDNA (right) complexes. The protein subunits and ssDNA binding surfaces are strikingly similar between the two proteins

The overall arrangement of the monomers within the SSB tetramer and the path of the ssDNA bound to the surface of the proteins in the SsbB·ssDNA complex strongly resembles that of SSB_{Eco} *or* $SsbA_{Hpy}$ bound to ssDNA (Raghunathan, Kozlov et al. 2000, Chan, Lee et al. 2009). Based on structural analysis of bacterial SSBs solved thus far, $SsbA_{Hpy}$, which plays an active role during vegetative growth and natural transformation, has a similar structure than SsbB. It is likely that both SsbA and SsbB have similar ternary structures.

4.1.4. SsbB* binding characterization:

To determine whether these C-terminal residues of SsbA play a significant role in RecO activation of RecA nucleation onto ssDNA, a hybrid ssbB-ssbA gene was constructed. A DNA segment encoding the last nine codons of ssbA, including the hexapeptide protein-binding motif $DDD_I/_LPF$, was fused to the 3'-end of the ssbB gene. This 122 codon-long *ssbB** gene expressed SsbB* protein, the full-length SsbB fused to the nine C-terminal residues of SsbA. SsbB* protein have high binding efficiency than the SsbB protein (see Table 5).

Table 6: SsbB* binding quantification

Substrate	DNA binding affinity (in nM)	
	SsbB*	
	- Mg^{2+}	+ Mg^{2+}
$polydT_{80}$ [a]	0.9±0.1	0.8±0.2
$ssDNA_{80}$ [a]	ND	ND
$polydT_{80}$ [b]	ND	ND

The Kd_{app} values (in nM) are the average of at least three independent experiments and are within a 10% standard error. [a]Proteins were incubated with the indicated substrate for 15 min at 37 °C in buffer F containing or not 10 mM $MgCl_2$. Samples were separated by 10 % PAGE, and the formation of protein/DNA complexes was quantified as described in Materials and Methods. [b]Proteins were incubated with the indicated substrate for 15 min at 37° C in buffer F lacking $MgCl_2$. The mixture was filtered through KOH-treated filters (millipore, type HAWP 45 mm), the filters dried and the amount of radioactivity bound to the filter was determined by scintillation counting. ND, not done.

4.2. Transformation efficiency analysis: Alternative pathways for genetic recombination

The centre of chromosomal transformation is the formation of a RecA nucleoprotein filament, because a null *recA* (Δ*recA*) strain blocks chromosomal transformation, but marginally (< 3-fold) affects, if at all, plasmid transformation (Dubnau and Cirigliano 1973, Canosi, Morelli et al. 1978, Alonso, Luder et al. 1991, Table 7). The $ssbA^-$ strain is not viable, hence its contribution on chromosomal and plasmid transformation cannot be assayed, removal of the C-terminal acidic tail render cells with a thermo sensitive phenotype that can be overcome by over-expression RecO (Costes, Lecointe et al. 2010). Since the absence of DprA reduced chromosomal transformation 10- to 100-fold (Berka, Hahn et al. 2002, Ogura, Yamaguchi et al. 2002, Tadesse and Graumann 2007) and the absence of RecO reduced plasmid transformation ~ 30-fold (Fernandez, Kobayashi et al. 1999), suggest that different mediators might recruit RecA onto SsbA- and/or SsbB-coated ssDNA, and a second strand annealing protein might facilitates plasmid transformation.

4.2.1. RecO role in genetic transformation

To determine whether RecO helps RecA to overcome the interference imposed by the SSB proteins for binding to ssDNA three strains were constructed including the null *recO dprA* (Δ*recO* Δ*dprA*) double mutant strain. The Δ*dprA* strain exhibited ~50- and ~40-fold reduction in chromosomal and plasmid transformation, respectively, whereas the Δ*recO* strain impaired plasmid transformation (~30-fold), but only marginally affected chromosomal transformation (<3-fold) (Table 7).

RecO, which is only 29% identical to the first 164 amino acids of the 255-residue $RecO_{Eco}$ protein, could mediate RecA loading onto SSB-coated ssDNA, but its role could be hindered by the redundancy of genetic recombination. It was previously shown that: i) RecA

focus formation during natural competence was significantly reduce in Δ*recO* competent cells (Kidane, Carrasco et al. 2009); ii) RecO loads RecA onto SsbA-coated ssDNA during recombinational repair (Carrasco, Manfredi et al. 2008, Manfredi, Carrasco et al. 2008); iii) RecO, which physically interacts with SsbA, catalyses single strand annealing that is needed during plasmid transformation (Kidane, Carrasco et al. 2009); and iv) $DprA_{Spn}$ accelerate ssDNA annealing of naked complementary ssDNAs more than 5-fold relative to the protein-free reaction (Mortier-Barriere, Velten et al. 2007).

Chromosomal and plasmid transformation were drastically impaired in Δ*recO* Δ*dprA* cells, but not abolished when compared to the Δ*recA* strain (Table 7). It is likely therefore that: i) RecO, in the absence of DprA, works as a RecA mediator contributing to RecA-mediated chromosomal transformation; ii) in the absence of both RecO and DprA, RecA might overcame the inference imposed by the SSB to bind and nucleate onto SSB-coated ssDNA, albeit with low efficiency; and iii) DprA plays an essential, but unknown role in plasmid transformation, because it was shown that *B. subtilis* DprA fails to catalyze DNA strand annealing (quoted in Claverys, Martin et al. 2009).

4.2.2. DprA is important for plasmid transformation

DprA is required for chromosomal transformation in different bacterial species, but its requirement for plasmid transformation is less clear. For example, plasmid transformation is marginally reduced if at all in $\Delta dprA_{Hpy}$ or $\Delta dprA_{Hin}$ competent cells (Karudapuram, Zhao et al. 1995, Ando, Israel et al. 1999), but it is >1000-fold reduced in the $\Delta dprA_{Spn}$ context (Bergé, Mortier-Barriere et al. 2003). In the latter case, the lack of the transforming capacity was attributed to the rapid degradation of the incoming ssDNA in $\Delta dprA_{Spn}$ as well as in $\Delta recA_{Spn}$ competent cells (Bergé, Mortier-Barriere et al. 2003). In *B. subtilis* competent cells, the half-life of incoming ssDNA was neither affected in the Δ*dprA* (Tadesse and Graumann 2007) nor in the Δ*recA* context (Dubnau and Cirigliano 1973). Chromosomal transformation is RecA dependent, whereas plasmid transformation is RecA independent (Table 7) (Kidane, Ayora et al. 2012).

Table 7. Effect of the absence of both RecO and DprA on genetic recombination

Relevant genotype	Normalised chromosomal transformation[a]	Normalised plasmid transformation[b]
rec$^+$	100	100
Δ*rec*A	<0.01 (<0.01)[c]	97 (95)[c]
*Δssb*B	26 (20-30)[c]	ND
ΔrecO	48 (45)[c]	3.0 (2.7)[c]
Δ*dprA*	1.7 (1 – 10)[c]	2.5 (1.6)[c]
Δ*rec*O Δ*dprA*	<0.1	<0.1
Δ*rec*O Δ*recA*	<0.01[c]	48[c]
Δ*dprA* Δ*recA*	0.01	0.5

[a]The yield of *met*$^+$ transformants (chromosomal transformation) and [b]pUB110 kanamycin-resistant transformants (plasmid transformation) was corrected for DNA uptake and cell viability and the values obtained normalised relative to that of the *rec*$^+$ strain, taken as 1. The results are the average of at least five independent experiments and are within a 10% standard error. [c]Between parentheses are the transformation frequencies of *rec*$^+$ and single mutant strains (Δ*recO*, Δ*dprA* and Δ*recA*) reported elsewhere (Ceglowski, Luder et al. 1990, Fernandez, Kobayashi et al. 1999, Berka, Hahn et al. 2002, Ogura, Yamaguchi et al. 2002, Tadesse and Graumann 2007) and brought here for direct comparison.

The absence of DprA or RecO resulted in a 40- and 30-fold reduction in plasmid transformation, respectively, but in the absence of both RecO and DprA chromosomal and plasmid transformation were blocked (Table 7) (Yadav, Carrasco et al. 2012). RecO has two activities (Yadav, Carrasco et al. 2012), and hypothesized that DprA might also have similar

activities: to recruit RecA onto a SSB-coated ssDNA complex and to catalyse SSA, an activity important for plasmid transformation.

To confirm the role of DprA in plasmid transformation we have separated the activities associated with DprA by constructing a Δ*dprA* Δ*recA* double mutant strain (Table 7). Chromosomal transformation was blocked in the Δ*dprA* Δ*recA* or Δ*recO* Δ*recA* context. The RecO defects in plasmid transformation were not due to the unavailability of ssDNA, because the absence of RecA suppressed the need for RecO during plasmid transformation (Table 7) (Yadav, Carrasco et al. 2012). Plasmid transformation was attenuated in Δ*dprA* Δ*recA* cells (Table 7), but not completely blocked because a few transformants were still observed. It is likely, therefore, that in the *recA*$^{+}$ background both SSA proteins, DprA or RecO, are required for plasmid transformation, but in the absence of RecA the relative concentrations of the SSB proteins is altered, and plasmid transformation was mainly carried out via DprA.

4.3. Biochemical characterization RecO role in genetic recombination

Firmicutes RecA bound to dATP is able to promote DNA strand exchange to form hybrid DNA *in vitro* without additional proteins, but RecA bound to dATP strictly requires accessory factor. These accessory factors can stimulate strand exchange. These factors can be divided into two broad classes: those that act before homology search by promoting assembly of RecA filaments, and those that act during homology search and strand exchange. Assembly factors can, in turn, be divided into two classes: the SSB proteins and the assembly "mediators", RecO and DprA. We focus on the roles of assembly factors that act before homology search.

4.3.1. SsbA, SsbB and RecO role during chromosomal transformation

4.3.1.1. SsbA, SsbB and SsbB* constrain and RecO facilitates RecA nucleation onto ssDNA

In both natural transformation and recombinational repair, RecA is required for binding to ssDNA in the first of the multi-step recombination processes. Yet, ssDNA is rarely present in the cell without SsbA (or perhaps SsbB); therefore, the dynamics of RecA and SsbA (SsbB) binding to the same ssDNA play an important role in understanding these processes. RecA nucleation onto ssDNA, and subsequent extension of RecA filaments can be monitored by measuring the rate of dATP hydrolysis under RecA-limiting conditions. The rate of hydrolysis of dATP also provides an indirect measure of the displacement of SsbA and/or SsbB from ssDNA by RecA. We used this approach to examine RecA nucleation and filament extension onto SsbA-, SsbB- or SsbA- and SsbB-coated ssDNA.

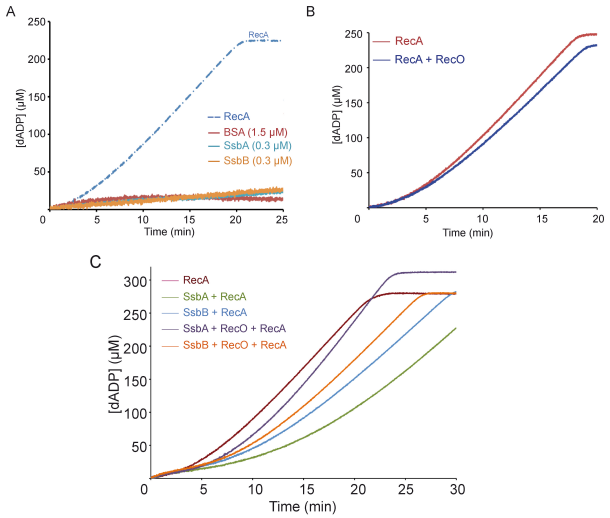

Figure 14. SsbA or SsbB plays a role in the rate-limiting nucleation of RecA and RecO activation. (A) SSBs controls to show absence of ATPase activity in proteins (B) The 3,199-nt ssDNA (10 μM in nt) was pre-incubated with RecO (100 nM) in buffer G containing 5 mM dATP. RecA (800 nM) was then added and the absorption measured for 20 min. (C) The 3199-nt ssDNA (10 mM in nt) was pre-incubated with SsbA or SsbB (300 nM) and then incubated or not with RecO (100 nM) in buffer G containing 5 mM dATP. Then RecA (800 nM) was added.

The purified SsbA or SsbB protein cannot hydrolyze dATP (Figure 14A), hence in our assays were measuring the RecA activity (Figure 14 B and 14C). The rate of RecA (one RecA/ 12-nt) nucleation onto naked ssDNA, and subsequent filament formation was biphasic, with a <5-min lag phase preceding establishment of the maximal hydrolysis rate (Figure 14C). Pre-binding of SsbA or SsbB (one SsbA or SsbB tetramer/ 33-nt) to ssDNA extended the RecA lag phase to ~ 11 or ~7 min, respectively (Figure 14C). This is consistent with competitive binding between RecA and SsbA or SsbB for the ssDNA, limiting RecA nucleation. Since the half-lives for both SsbA-ssDNA and SsbB-ssDNA complexes with polydT$_{80}$ or longer ssDNAs were longer than the time of reaction (see Figure 11), nucleated RecA is likely displacing SsbA and SsbB during filament extension, albeit at a low rate. Similar results are seen with SSB$_{Eco}$ and RecA$_{Eco}$ in that SSB$_{Eco}$ delays nucleation of RecA$_{Eco}$ onto SSB$_{Eco}$-coated ssDNA (~20 min lag time).

4.3.1.2. RecA nucleacles on RecO·SsbA·ssDNA·SsbB complex more efficient than on RecO·SsbA·ssDNA or RecO·ssDNA·SsbB complexes

To determine the effect of both SsbA and SsbB on RecA nucleations onto ssDNA, both proteins were co-assembled onto ssDNA (creating an SsbA-ssDNA-SsbB complex) and RecA-mediated dATP hydrolysis analysed (Figure 15A).

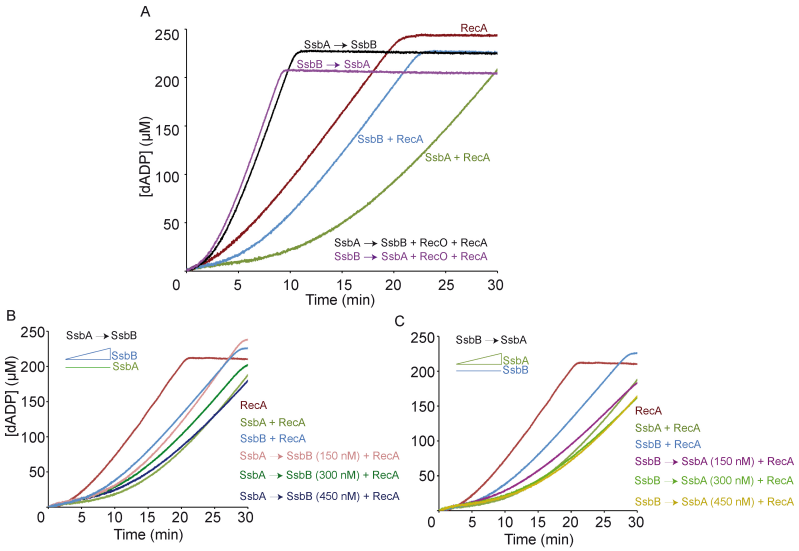

Figure 15. The ssDNA was pre-incubated with SsbA, SsbB or with SsbA and then with SsbB (SsbA→SsbB) or vice versa (SsbB→SsbA) in buffer G containing 5mM dATP. RecO was added and incubated for 5 min. RecA was then added and the absorption measured for 30 min **(A)**. SsbA or SsbB plays a role in the rate-limiting nucleation of RecA and RecO in the activation. The 3,199-nt ssDNA was pre-incubated with a fix amount of SsbA (300 nM) and increasing concentrations of SsbB (150, 300 and 450 nM) **(B)** or fix amount of SsbB (300 nM) and increasing concentrations of SsbA (150, 300 and 450 nM) **(A)** in buffer G containing 5 mM dATP. Then RecA (800 nM) was added and the absorption measured for 30 min.

When ssDNA was pre-incubated with SsbB (one SsbB tetramer per 33-nt) followed by addition of excess of SsbA, the RecA nucleation time onto ssDNA was increased to levels comparable to SsbA alone (Figure 15C). However, the same was not true for the addition of excess amount of SsbB to saturating amounts of SsbA (one tetramer per 33-nt) pre-bound to ssDNA; there was no decrease in RecA nucleation time (Figure 15A). It is likely that in mixed SsbA-ssDNA-SsbB complexes, SsbA exerts a dominant negative effect on RecA nucleation over SsbB (Figure 15A).

Since SsbA has a significant effect on RecA nucleation moderated by interacting with RecO, we predicted that RecO could dislodge both SsbA and SsbB bound to ssDNA at a different rate than either SsbA or SsbB alone. To test this hypothesis, SsbA was pre-incubated with ssDNA and SsbB added (or *vice versa*) followed by addition of RecO (one RecO per 100-nt). RecA-mediated dATP hydrolysis was then measured for the heterologous SSB-coated ssDNA (Figure 15A). Since the second SSB protein was added after the first was already in complex with ssDNA, formation of heterotetrameric proteins was unlikely. A co-assembled SsbA-ssDNA-SsbB complex markedly reduced the rate-limiting RecA nucleation to <2 min (Figure 15A). This co-assembly of SsbA and SsbB might enable RecO to recognize SsbA and carry out the limited release of SsbA or both SsbA and SsbB from ssDNA, subsequently loading RecA more efficiently. In addition, RecA displaced the SSB proteins from the heterologous complex more effectively than SsbA or SsbB alone, suggesting that the functional interaction between SsbB and RecA might be facilitated by the presence of SsbA, RecO or both.

4.3.1.3. RecO does not contribute to facilitates RecA nucleation onto SsbB*-coated ssDNA

The genetic data suggest that RecO can act as an alternate RecA mediator and the biophysical information that RecO physically interacts with SsbA. It was hypothesized that the acidic C-terminal end of SsbA was involved in the interaction with RecO (see Hobbs, Sakai et al. 2007). To test this hypothesis a DNA segment encoding the last nine codons of *ssbA*, including the hexapeptide protein-binding motif DDD$^{I}/_{L}$PF (Lu and Keck 2008), was fused to the 3'-end of the *ssbB* gene. This 122 codon-long *ssbB** gene expressed SsbB*, the full-length SsbB fused to the nine C-terminal residues of SsbA. The SsbB* variant was used

to determine the effect of adding RecO to the RecA dATPase assays (Figure 16A).

Purified SsbB* decreased RecA nucleation onto SsbB*-coated ssDNA compared to SsbB·ssDNA alone. Also, addition of RecO prior to RecA moderately assisted RecA loading onto SsbB*·ssDNA (Figure 16A). Interestingly the addition of the C-terminal residues on SsbB* did not show the same response to RecO as SsbA even though SsbB* bound ssDNA with an ~1.7 fold higher affinity than SsbB (Tables 5 and 6).

The C-terminal end of SsbB* was solvent exposed as shown by sensitivity to trypsin proteolysis (Figure 16B and 16C). It is likely that SsbA does not solely interact with RecO through the nine C-terminal-most residues; this is consistent with the observation that SSB_{Tth} interacts with $RecO_{Tth}$ through more than just its C-terminal region (Inoue, Nagae et al. 2011).

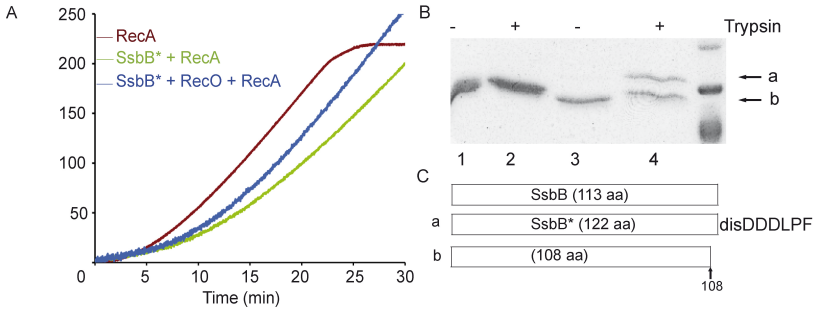

Figure 16. RecO dose not interact with SsbB*. (A) The 3199-nt ssDNA (10 mM in nts) was pre-incubated with SsbB* (300 nM) and then incubated or not with RecO (100 nM) in buffer G containing 5 mM dATP. Then RecA (800 nM) was added and the absorption measured for 30 min. **(B)** Partial proteolysis assays. SsbB (lanes 1 and 2) or SsbB* (lanes 3 and 4) were pre-incubated (+) or not (-) with ssDNA and then ProK was added and the mixtures were analysed by 20% SDS-PAGE. The polypeptides were isolated, subjected to partial proteolysis and mass spectrometry. **(C)** Representations of mass spectrometry products shown for bands **a** and **b** of SsbB* in (B)

4.3.1.4. RecO facilitates RecA-mediated DNA strand exchange in the presence of both SsbA and SsbB

SsbA or SSB_{Eco} pre-bound to ssDNA inhibits RecA nucleoprotein filament formation and dATP hydrolysis, but when added after RecA, SSBs generally aid RecA-mediated DNA strand exchange by melting inhibitory secondary structure in the ssDNA substrate and coating the displaced strand of the three strand reaction (reviewed in Kowalczykowski, Dixon et al. 1994, Cox 2007, Manfredi, Carrasco et al. 2008). To better understand the effects of the co-assembled SsbA·ssDNA·SsbB complex on RecA function not simply binding, we next examined the effects of adding either SSB protein to RecA-catalysed DNA strand exchange reactions.

In the absence of SsbA or SsbB, limiting RecA concentrations catalysed dATP-dependent strand exchange between circular ssDNA (*css*) and a linear dsDNA (*lds*), converting ~10% of the homologous ldsDNA into joint molecules (*jm*) and the final nicked-circular (*nc*) product during a 60-min reaction (Figure 17A, lanes 2 and 9). The addition of half-saturating to saturating SsbA or SsbB (1 tetramer/ 66-, 40- and 33-nt), added prior to RecA significantly stimulated RecA strand exchange (~3- and 2-fold, respectively) as judged by the accumulation of dATP-dependent *jm* intermediates and *nc* products (Figure 17A, lanes 3-5 and 6-8).

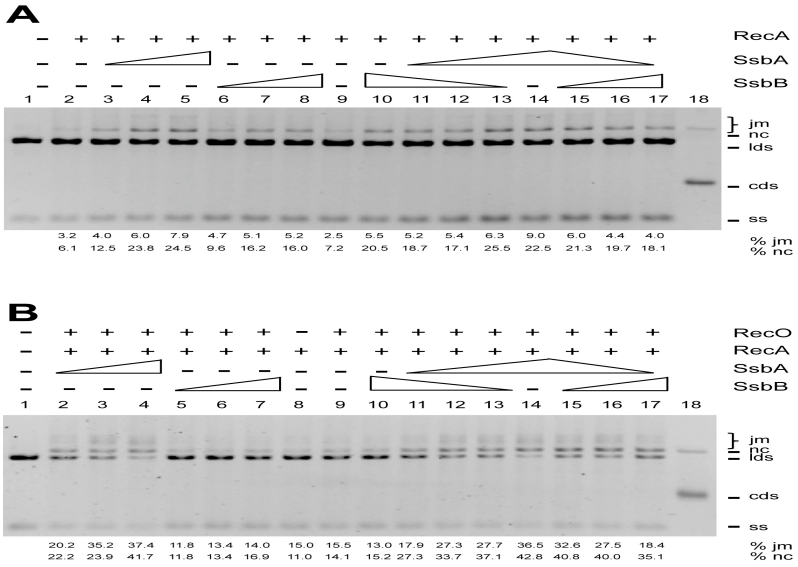

Figure 17. RecO facilitates RecA loading onto SsbA·ssDNA or SsbA·ssDNA·SsbB. **(A)** Circular ssDNA (10 mM in nt) and homologous *Kpn*I-linearised dsDNA (20 mM in nt) were pre-incubated with increasing concentrations of SsbA or SsbB (150, 250, 300 nM; lanes 2-5, 6-8) or decreasing concentrations of SsbB and increasing concentrations of SsbA or *vice versa* (150, 250, 300 and 450 nM; lanes 10-14, 15-17) for 5 min at 37ºC in buffer G containing 2 mM dATP. Then a constant amount of RecA (800 nM, lanes 2-17) was added and the reaction incubated for 60 min at 37ºC. **(B)** Circular ssDNA and homologous linear dsDNA were pre-incubated with increasing concentrations of SsbA or SsbB (150, 250, 300 nM; lanes 2-4, 5-7) or decreasing concentrations of SsbB and increasing concentrations of SsbA or *vice versa* (150, 200, 300 and 450 nM; lanes 10-14, 15-17) for 5 min at 37ºC in buffer D containing 2 mM dATP. The complex was incubated with a constant amount of RecO (100 nM, lanes 2-17) for 5 min at 37ºC, followed by addition of a constant amount of RecA (700 nM, lanes 2-17) and incubated for 60 min at 37ºC. The products of the reactions were deproteinised, separated and monitored by 0.8% PAGE with ethidium bromide. The position of the bands corresponding to *css*, *lds*, *nc*, *jm* and *ccc* are indicated. +/- denotes the presence or absence of the indicated protein. Lane 18, indicates the partially nicked dsDNA substrate that was used to mark the position of the *nc* substrate. The percentage of *jm* intermediates and *nc* products are shown below each lane.

The presence of SsbA, even in limited quantities, along with SsbB also enhanced strand exchange, ~30% of the ldsDNA substrate was converted to *jm* intermediates and *nc* products (Figure 17A, lanes 10-17). This result suggests that SsbA plays a major role in facilitating RecA-mediated strand exchange than does SsbB, though both could be enhancing strand exchange by removing ssDNA secondary structure and sequestering the displacement of the newly generated ssDNA.

As previously reported, the accumulation of *jm* intermediates increases with the presence of RecO, suggesting that RecO modulates the extent of RecA-mediated DNA strand exchange (Manfredi, Carrasco et al. 2008). To test whether the RecO mediator acts by targeting RecA using SsbA or SsbB, RecA-mediated strand exchange in the presence of RecO and SsbA, SsbB or both was measured. RecO (1 RecO per 100-nt) significantly increased the accumulation of *jm* intermediates and *nc* product with SsbA (Figure 17B, lanes 2-4) as compared to the absence of RecO (Figure 17A, lanes 3-5). The addition of RecO to SsbB·ssDNA did not stimulate RecA-mediated accumulation of *nc* products (Figure 17B, lanes 5-7), and only increased the accumulation of *jm* to a similar extent compared to RecO alone (see Manfredi, Carrasco et al. 2008). The hybrid protein SsbB* showed similar results to SsbB (data not shown).

In the presence of both SsbA and SsbB proteins in the form of the SsbA·ssDNA·SsbB complex, addition of RecO increased RecA-mediated DNA strand exchange when SsbA was in excess compared to SsbB, independent of the order of addition (Figure 17B, lanes 10-17). RecO interaction with SsbA likely enables RecA utilization of SsbA·ssDNA and SsbA·ssDNA·SsbB and promotes RecA re-invasion of the displaced ssDNA as deduced by the accumulation of *jm* intermediates, but re-invasion cannot take place on SsbB-coated ssDNA due to the lack of RecO interaction. Similarly, RecO is unable to overcome the inhibitory effect of Ssb_{SPP1} or SSB_{Eco} when added before RecA (Carrasco, Manfredi et al. 2008, Manfredi, Carrasco et al. 2008).

4.3.2. Biochemical characterization of SsbA, SsbB and RecO for plasmid transformation

4.3.2.1. SsbA facilitates RecO-mediated DNA strand annealing

Plasmid transformation, which is a RecA-independent event, in *B. subtilis* requires RecO (Table 7). RecO localizes to the entry pole when oligomeric plasmid DNA, which can self-anneal, enters the cell. In addition, SsbA-coated ssDNA facilitates RecO-mediated annealing of complementary ssDNA strands. To study the contribution of SsbA and SsbB on RecO-dependent plasmid transformation, the effects of SsbA, SsbB, or both on the rate of RecO-mediated SSA were measured. When compared with the absence of SSBs.

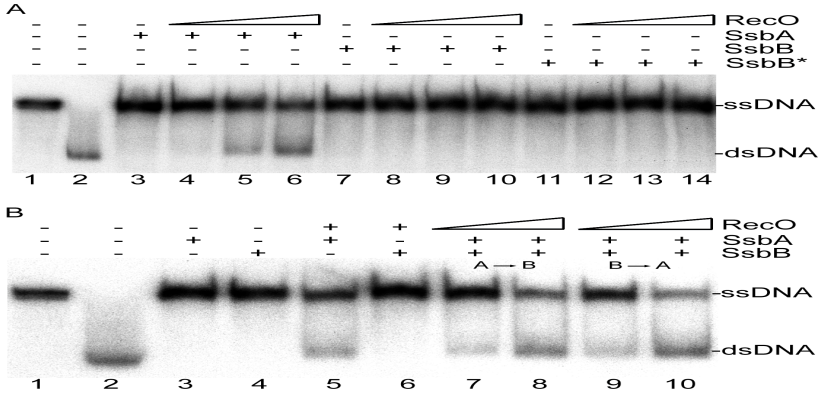

Figure 18. RecO anneals complementary strands complexed with SsbA protein. (A) Heat-denatured 440-nt long [α-^{32}P]-ssDNA (7 μM in nt) was quickly cooled and pre-incubated with a fix amount of SsbA, SsbB or SsbB* (100 nM) for 10 min at 30° C in buffer H, and then incubated with increasing concentrations of RecO (1, 2 and 3 μM) for 60 min at 30° C. (B) The heat denatured ssDNA was pre-incubated with a fix amount of SsbA, SsbB, SsbA followed by SsbB (lanes 7 and 8) or vice versa (lanes 9 and 10) (100 nM) for 10 min at 30° C in buffer E, and then incubated with a fix amount (2 μM, lanes 5 and 6) or increasing concentrations of RecO (1 and 2 μM, 7–10) for 60 min at 30° C in buffer H. Lane 2, heat-denatured ssDNA was slowly cold down (spontaneous annealing). The products of the reactions were deproteinised, separated by 6% PAGE and monitored by using a Geldoc (BioRad) system.

When compared with the absence of SSBs, the addition of SsbA, SsbB or SsbB* blocked spontaneous strand annealing of complementary homologous 440-nt ssDNA (Figure 18A, lanes 3, 7 and 11). Only SsbA facilitated RecO-mediated annealing of the complementary ssDNA molecules (Figure 18A, lanes 5 and 6). SsbB did not stimulate RecO-mediated strand annealing nor was the C-terminal end of SsbA, in the context of SsbB*, sufficient to contribute in the interaction with RecO and stimulate activity (Figure 18A, lanes 8–10 and 12–14).

SsbA as part of the heterologous SsbA-ssDNA-SsbB complex facilitated RecO-mediated DNA strand annealing (Figures 18B, lane 8 and 10), again suggesting the significance of the functional interaction between SsbA and RecO.

The SsbB·ssDNA complex inhibits RecO-mediated annealing of complementary strands, whereas SsbA·ssDNA recruits RecO to form a ternary SsbA·RecO·ssDNA (Manfredi, Suzuki et al. 2010). In the co-assembled SsbA·ssDNA·SsbB complex, RecO interaction with SsbA leads to the formation of bridged structures (Manfredi, Suzuki et al. 2010), rather than mutually exclusive interactions, either decreasing the half-life of SsbA- and SsbB-coated ssDNA or altering the structure of ssDNA and facilitating the dissociation of both SsbA and SsbB from ssDNA. Either of these avenues might result in faster net disassembly of both SsbA and SsbB, and the SsbA-mediated assembly of RecO promotes DNA strand annealing (during plasmid transformation) (Figure 18B).

4. 4. Effect of nucleotide cofactor on RecA activities

In the presence of dATP, $RecA_{Eco}$ binds ssDNA more tightly, and invades more secondary structure resulting in fast polymerization onto ssDNA or prevents a net end-

dependent disassembly than it can in the presence of ATP (Menetski and Kowalczykowski 1989, Shan, Bork et al. 1997). Firmicutes RecA does not efficiently nucleates onto SSB-coated ssDNA (Lovett and Roberts 1985, Steffen and Bryant 1999, Steffen, Katz et al. 2002, Carrasco, Manfredi et al. 2008, Grove, Anne et al. 2012).

4. 4.1. RecA loading onto ssDNA shows a different preference for a nucleotide cofactor

To investigate why ATP does not efficiently support RecA polymerization onto ssDNA we used ssDNA-dependence dATP or ATP (denoted as [d]ATP) hydrolysis. Briefly, the hydrolysis of the nucleotide cofactor was used as an indirect measure of RecA nucleation and filament assembly and disassembly (filament extension) onto ssDNA. The rate of RecA nucleation onto naked circular ssDNA was not significantly affected by the concentration of RecA; however, the rate of (d)ATP hydrolysis correlated with the amount of RecA bound to ssDNA (Yadav, Carrasco et al. 2012). The lag phase of RecA ($RecA_{Eco}$) nucleation (rate limiting) derives from the time intercept of the linear regression based on the steady state rate of hydrolysis of the nucleotide cofactor (see Hobbs, Sakai et al. 2007, Yadav, Carrasco et al. 2012).

In the presence of dATP and 1 RecA monomer/ 12-nt, the rate of RecA nucleation onto naked ssDNA and subsequent filament formation was biphasic, with a slow nucleation step (3 - 5 min lag phase) preceding establishment of the maximal hydrolysis rate (end-dependent filament assembly and disassembly) (Figure 14C) (Yadav, Carrasco et al. 2012). In the presence of limiting RecA concentrations (1 RecA/ 25-nt), RecA nucleation onto naked ssDNA did not seem show a significant delay in nucleation, because RecA·ssDNA filament assembly and subsequent disassembly was significantly reduced (Figure 19). The rate of ssDNA-dependent dATP hydrolysis by RecA was similar to previously observed values (Lovett and Roberts 1985, Steffen and Bryant 1999), and the turnover number of Firmicutes RecA was similar to $RecA_{Eco}$ under similar experimental conditions (see Steffen and Bryant 1999).

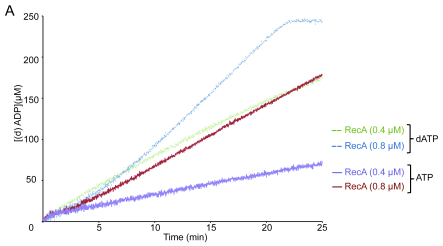

Figure 19. RecA nucleation effect in presence of ATP or dATP. *A*, circular 3,199-nt ssDNA (10 μM in nt) was incubated with RecA (0.4 and 0.8 μM) for variable time at 37 ºC in Buffer G containing 5 mM ATP or dATP. The reaction was carried out under standard condition as described in Experimental procedures, and the ssDNA-dependent dATPase or ATPase ([d]ATPase) activity was measured for 25 min.

The rate of RecA nucleation onto naked ssDNA in the presence of ATP showed a different shape than in the presence of dATP. RecA nucleation apparently did not shown a significant lag phase (~0.5 min) in the presence of ATP, but RecA·ssDNA filament assembly and disassembly was slow and reduced compared to RecA in the presence of dATP (Figure 19). The rate of ssDNA-dependent ATP hydrolysis by RecA was significantly lower that with dATP as previously documented (Lovett and Roberts 1985, Steffen and Bryant 1999, Carrasco, Manfredi et al. 2008, Manfredi, Carrasco et al. 2008).

4.4.2. Effect of the pH on RecA nucleation and filament growth

RecA nucleation is rate limiting (3 – 5 min lag phase) in the presence of dATP (see Figures 14C and 19), suggesting that RecA·dATP·Mg^{2+} undergoes a conformational change for nucleation. Alternatively, this delay can be explained if we assumed that RecA has a conformation or oligomeric state that is optimal for polymerization, but not for RecA

nucleation. For example, it has been shown that $RecA_{Eco}$ nucleates as a dimer (Bell, Plank et al. 2012) and extent as a monomer (Joo, McKinney et al. 2006, Bell, Plank et al. 2012) or nucleation and filament extension requires a small oligomer (van Loenhout, van der Heijden et al. 2009). To investigate whether a conformational changes of RecA facilitates nucleation and filament assembly/disassembly, the pH of the reaction was varied and the RecA states monitored by measuring the ssDNA-dependent RecA (d)ATPase activity.

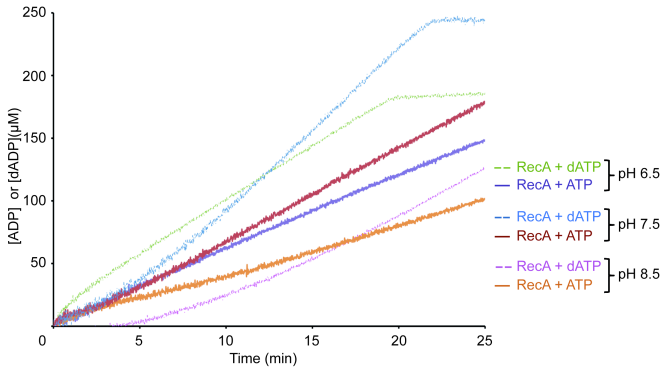

Figure 20. RecA nucleation effect in presence varying pH circular ssDNA was incubated RecA (0.8 μM) in Buffer with (pH 6.5, 7.5 or 8.5) containing 5 mM ATP or dATP. Then the ssDNA-dependent (d)ATPase activity was measured for 25 min.

The intracellular pH is highly regulated and maintained in the range of 7.4 to 7.8 *in vivo*, and RecA has an isoelectric point of ~5.0, which suggests that the protein will have a net negative charge at physiological pH. In the presence of dATP, RecA nucleation is rate limited, but assembly of filaments in the dATP conformation is much faster than assembly in the ATP conformation at physiological pH (Figure 20). At pH 6.5, RecA nucleation onto naked ssDNA did not show a lag phase when compared to pH7.5 (Figure 20). However, at high pH, RecA nucleation was further delayed (1.7-fold), and the rate of RecA-mediated dATP hydrolysis was significantly reduced (Figure 20).

In the presence of ATP, RecA nucleation onto naked ssDNA did not show an apparent lag phase, and it was marginally affected by varying the pH (Figure 20). The rate of RecA-mediated ATP hydrolysis was similar at pH 6.5 and 7.5, but the RecA dynamic assembly onto and disassembly from ssDNA was significantly reduced at pH 8.5 when compared to pH 7.5. It is likely that ionic interactions are important for the assembly at acidic or physiological pHs, that are optimal for RecA activity. Similarly, $RecA_{Eco}$ is strongly stimulated by reducing the pH to 6.5, where it is predominantly a dimer in solution (McEntee, Weinstock et al. 1981).

4.4.3. SSB inhibits RecA nucleation onto naked ssDNA

ssDNA depended RecA-mediated dATP hydrolysis is reduced. When dATP is replaced by ATP the nucleotide hydrolysis is blocked (Carrasco, Manfredi et al. 2008). ssDNA-dependent hydrolysis of (d)ATP in the presence of SsbA- or SsbB-coated ssDNA was re-evaluated (Figure 21). In our experiments, RecA is limiting over ssDNA (1 RecA/ ~12-nt) to measure the role of the SSB protein (1 SSB/ 33-nt) in RecA nucleation, and RecA·ssDNA filament formation. In the presence of dATP the rate of RecA nucleation onto naked ssDNA and subsequent filament formation was biphasic, with 4 - 5 min lag phase, proceeding establishment of the maximal hydrolysis rate (Figure 21) (Carrasco, Manfredi et al. 2008, Manfredi, Carrasco et al. 2008, Yadav, Carrasco et al. 2012). SsbB interacts with RecA (SSB_{Eco}) *in vivo* (Kramer, Hahn et al. 2007), suggesting that a SsbB·RecA interaction might help RecA nucleation onto SsbB·ssDNA complexes. SsbA or SsbB (1 SSB/ 33-nt) bound to ssDNA extended the RecA lag phase to ~11 min or ~7 min, respectively, in the presence of dATP and reduced RecA·ssDNA filament formation (Figures 21A and 21B) (Carrasco, Manfredi et al. 2008, Manfredi, Carrasco et al. 2008, Yadav, Carrasco et al. 2012).

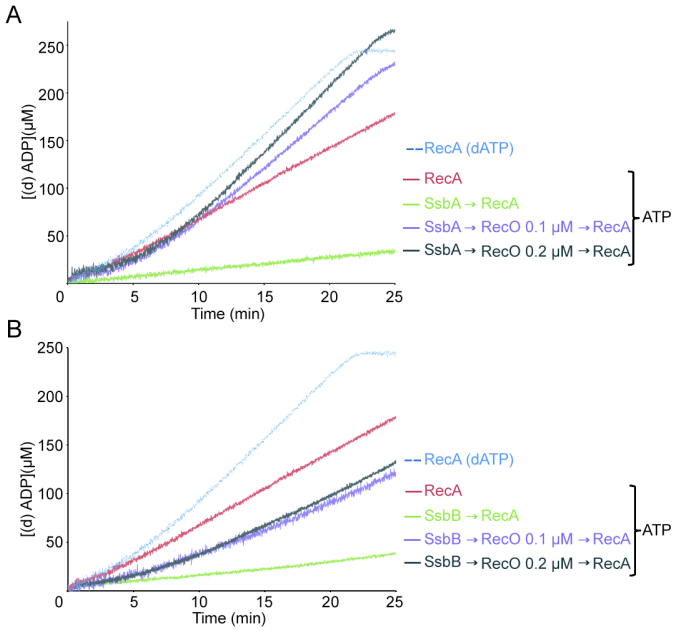

Figure 21. Effect of SsbA, SsbB and RecO on RecA nucleation in presence of ATP or dATP. *A*, circular ssDNA (10 μM in nt) was pre-incubated with SsbA (0.3 μM) for 5 min at 37 °C in buffer G containing 5 mM ATP or dATP, and then RecO (0.1 or 0.2 μM) was incubated with the preformed SsbA·ssDNA complexes for 5 min at 37 °C. Finally RecA (0.8 μM) was added, and the absorption was monitored for 25 min. *B*, circular ssDNA (10 μM in nt) was pre-incubated with SsbB (0.3 μM) for 10 min at 37 °C in buffer G containing 5 mM ATP or dATP, and then RecO (0.1 or 0.2 μM) was incubated with the preformed SsbB·ssDNA complexes for 10 min at 37 °C. Finally RecA (0.8 μM) was added, and the absorption was monitored for 25 min.

4.4.4. SSB inhibits and RecO facilitates RecA loading onto SsbA-coated ssDNA

When ATP was provided in place of dATP a different outcome was observed. RecA nucleation and filament growth onto SsbA- or SsbB-coated ssDNA was blocked when compared to naked ssDNA (Figures 14C, 21A and 21B) (Carrasco, Manfredi et al. 2008). Since, RecA·ATP·Mg^{2+} nucleation on SsbA·ssDNA or SsbB·ssDNA complexes was inhibited to a similar extent we have to assume RecA in the ATP might not interact with SsbB. Alternatively, the putative SsbB·RecA interaction might play no role on RecA nucleation.

In vitro RecA·dATP·Mg^{2+} nucleates on the RecO·ssDNA complexes with similar efficiency that on naked ssDNA (Figure 14B) (Manfredi, Carrasco et al. 2008). We have investigated the effect of the RecO mediator on RecA nucleation and RecA·ssDNA filament extension onto ssbA- or SsbB-coated ssDNA in the presence of ATP (Figure 21). SsbA and RecO or SsbB and RecO (1 SSB/ 33-nt and 1 RecO/ 100-nt) did not exhibit ATP hydrolysis activity when compared to the mock reaction in the absence of both proteins or when BSA was added instead of both proteins (data not shown). Therefore, the hydrolysis of ATP observed in our experiments can be solely attributed to the presence of RecA.

RecA nucleation and RecA·ssDNA filament assembly, and subsequent disassembly onto SsbA- or SsbB-coated ssDNA were inhibited in the presence of ATP (Figure 21A and 21B). At low ratios (1 RecO / 100-nt), RecO significantly facilitated RecA nucleation onto SsbA-coated ssDNA, and RecO stimulated RecA filament formation (Figure 21A). It is likely that RecO, upon interaction with SsbA, promotes a limited dislodging of SsbA from the SsbA·ssDNA complex, and significantly facilitates RecA·ATP·Mg^{2+} nucleation and filament growth (Figure 21A).

Addition of RecO (1 RecO/ 50- to 100-nt) partially reversed the negative effect exerted by SsbB on RecA nucleation. In the presence of SsbB and RecO, the lag time of RecA nucleation onto SsbB·ssDNA was extended >3-folds when compared to RecA nucleation onto naked ssDNA, but RecO allowed RecA to form RecA·ssDNA filaments when compared to RecA onto SsbB-coated ssDNA (Figure 21B).

4.4.5. RecO facilitates RecA loading onto naked ssDNA

Previously it has been shown that: i) RecO physically interacts with SsbA (Manfredi, Carrasco et al. 2008), but it does not interact with SsbB in solution (Yadav, Carrasco et al. 2012). RecO (*Mycobacterium smegmatis*, $RecO_{Msm}$) does not directly interact with the acidic C-terminal tail of SsbA (Yadav, Carrasco et al. 2012, Gupta, Ryzhikov et al. 2013); ii) RecO overcomes the negative effect exerted by SsbA (or both SsbA and SsbB) and facilitates RecA loading onto SsbA-coated ssDNA in the presence of dATP (Manfredi, Carrasco et al. 2008, Yadav, Carrasco et al. 2012); iii) RecO ($RecO_{Msm}$) contains a tetra-cysteine motif that is missing in $RecO_{Eco}$ (Gupta, Ryzhikov et al. 2013). The effect of the RecO mediator on RecA·ATP·Mg^{2+} nucleation and RecA·ssDNA filament extension onto naked ssDNA (in the presence or absence of Zn^{2+} metal ion) was monitored (Figure 22).

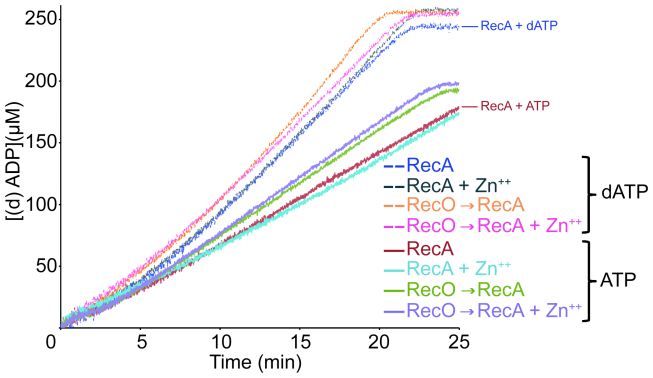

Figure 22. Effect of Zn^{2+} on RecA nucleation in presence of ATP or dATP and RecO. Circular ssDNA (10 μM in nt) was pre-incubated or not with RecO (0.1 μM) for 5 min at 37 °C in buffer G containing 5 mM ATP or dATP and with or without 10 μM $ZnSO_4$. Then RecA (0.8 μM) was added, and the absorption was monitored for 25 min.

In the absence of Zn^{2+}, RecO significantly stimulated RecA·ssDNA filament formation onto naked ssDNA without affecting RecA nucleation in the presence of ATP and in minor extent in the presence of dATP (Figure 22). It can be hypothesized that RecO binds ssDNA and produces a local torsion which increase the distance between adjacent bases similarly to $RecA_{Eco}$·ATP·Mg^{2+} (seeMasuda, Ito et al. 2009) that could then serves as RecA nucleation sites. Alternatively, the RecO and RecA interaction induces a conformational change in the latter, making it proficient for stable assembly on ssDNA. However, a direct RecO interaction with RecA was not detected in our experiments (Manfredi, Carrasco et al. 2008). Similarly, a complex between $RecA_{Eco}$ and $RecO_{Eco}$ or $RecOR_{Eco}$ was not observed (Umezu and Kolodner 1994).

The presence of Zn^{2+} (10 μM), did not significantly affected RecA nucleation and filament growth (Figure 22). RecO-mediated RecA·ATP·Mg^{2+} nucleation and filament growth was indistinguishable in the absence or presence of Zn^{2+} (Figure 22). Similar results were observed when ATP was replaced by dATP (Figure 22), suggesting that under the experimental conditions used, Zn^{2+} does not contribute to RecA nucleation or filament formation onto naked ssDNA. Alternatively, RecO in the Zn^{2+} bound form, in concert with RecR·Zn^{2+} (Ayora, Stiege et al. 1997, Ayora, Stiege et al. 1997) and RecF·ATP·Mg^{2+} (Ayora and Alonso 1997), contribute to RecA loading onto SsbA-coated ssDNA in *B. subtilis in vivo*.

4.4.6. RecO facilitates RecA nucleation onto SsbA- and SsbB-coated ssDNA

In the presence of dATP, RecA can nucleate on the quaternary RecO·SsbA·ssDNA·SsbB complexes more efficiently than on the ternary RecO·SsbA·ssDNA complexes (Figure 23), suggesting that SsbA facilitates RecO loading onto SsbA·ssDNA complexes (Manfredi, Suzuki et al. 2010). Then, RecO facilitates dislodging of both SSB proteins and RecA·dATP·Mg^{2+} nucleation onto SsbA·ssDNA·SsbB complexes. Finally, RecA·dATP·Mg^{2+} can displace both SSB proteins from ssDNA (Yadav, Carrasco et al. 2012). To investigate the effect of both SSB proteins on RecA in the presence of ATP, the ssDNA was pre-incubated with a fix amount of SsbB (1 SsbB/ 33-nt) and with variable amounts of SsbA. Then RecO and subsequently RecA were added and RecA loading onto ssDNA was indirectly measured (Figure 23). SsbB (or both SsbA and SsbB) blocked RecA·ATP·Mg^{2+} nucleation onto ssDNA to a similar extent (Fig 21A and 21B). RecO fully reversed the negative effect exerted by SsbB bound to ssDNA on RecA nucleation in the presence of limiting SsbA (1 SsbA/ 166-nt) concentrations. The presence of RecO significantly decreased the half time to reach the steady state of ATP hydrolysis estimated in ~13 min when compared to its absence that was estimated in ~21 min (Figure 23).

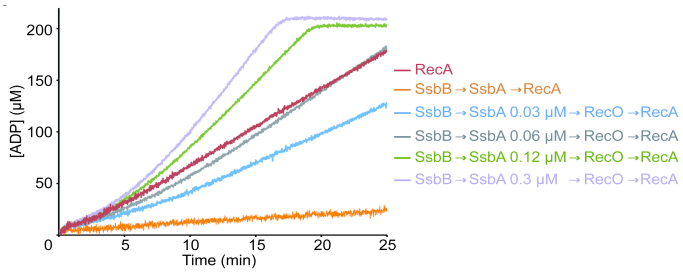

Figure 23. RecA efficiently nucleates in the SsbB·ssDNA·RecO complexes if SsbA in presence of ATP. Circular ssDNA (10 μM in nt) was pre-incubated with a fixed concentration of SsbB (0.3 μM) and SsbA (0.3 μM), or a fixed concentration of SsbB (0.3 μM) and a variable SsbA (0.06 to 0.3 μM) concentration for 5 min at 37 °C in buffer G containing 5 mM ATP. RecO (0.1 μM) was added, and 5 min later RecA (0.8 μM). The absorption was monitored for 25 min.

In the presence of stoichiometric amounts of SsbA and SsbB, the presence of RecO significantly reduced the nucleation time of RecA (Figure 23). Adding increasing SsbA concentration, SsbB and RecO significantly decreased the half time to reach the steady state of RecA-mediated ATP hydrolysis with higher efficiency than in the absence of SsbB (Figure 23), suggesting that the high RecA·ATP·Mg^{2+} rate of RecA·ssDNA filament formation cannot be attributed to a simple replacement of SsbB by SsbA from the ssDNA. Furthermore, RecA could nucleate and growth on RecO·SsbA·ssDNA·SsbB complexes in the presence of ATP as efficient as in the presence of dATP (Figure 23).

4.4.7. RecO and SsbA facilitates RecA-mediated DNA strand exchange

RecA cannot catalyze ATP-dependent accumulation of *jm* intermediates (or DNA pairing) and DNA strand exchange (accumulation of *nc* products). We can hypothesize that ATP altered the polarity of strand exchange, and the *jm* formed are not converted to *nc* products. $RecA_{Eco}$-mediated DNA strand exchange is slightly more efficient when the 3'-end of the linear complementary strand pairs with the circular ssDNA, although *jm* intermediates are efficiently formed with the 5'-end of the linear duplex, but are generally not converted to recombination products (Cox and Lehman 1981, Rosselli and Stasiak 1990, Konforti and Davis 1992).

In the presence of dATP, sub-saturating amounts of SsbA stimulated dATP-dependent

strand exchange when sub-saturating amounts of RecA were used, and the strand exchange reaction was further stimulated by the addition of RecO (Figures 17A and 17B). When the experiments were performed with ATP instead of dATP, no strand exchange products were observed when SsbA or SsbB was added prior RecA (Figure 24, lanes 2-8), but the addition of SsbA and RecO, before RecA, stimulated ATP-dependent strand exchange (Figure 24, lanes 9-10).

These results altogether show that: (i) unlike RecA·dATP·Mg^{2+}, sub-saturating concentrations of RecA·ATP·Mg^{2+} fail to displace SsbA or SsbB from ssDNA and strand exchange strictly requires RecO (Figure 17 and 24); and (ii) RecA can interact with the RecO·ssDNA·SsbA complexes and the linear homologous template and catalyze DNA strand exchange in the presence of dATP or ATP.

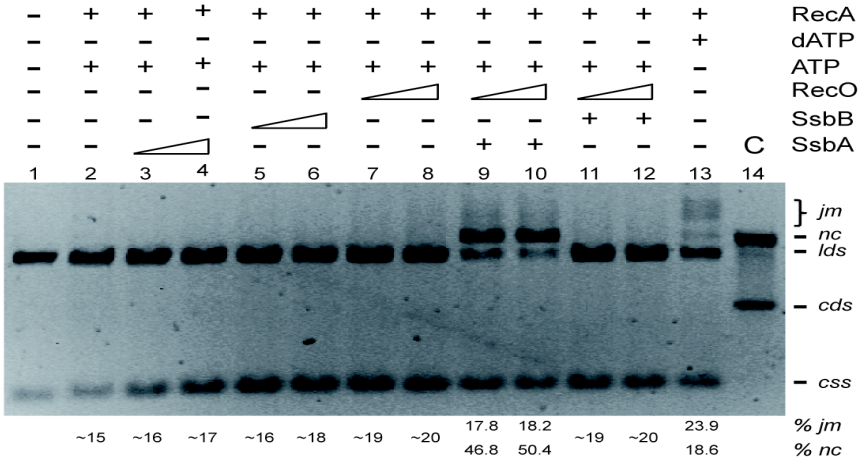

Figure 24. RecA-promoted DNA strand exchange in the presence of SsbA, RecO and different nucleotide cofactors. circular ssDNA (10 μM in nt) and homologous linear dsDNA (20 μM in nt) were pre-incubated with increasing concentrations of SsbA (0.15 and 0.3 μM), SsbB (0.15 and 0.3 μM) or RecO (0.2 and 0.4 μM), SsbA and RecO or SsbB and RecO for 10 min at 37 °C in buffer G containing 2 mM ATP or dATP, and then RecO was added to the preformed SsbA·ssDNA or SsbB·ssDNA complexes and incubated for 10 min at 37 °C. Finally, RecA (1 μM) was added and the reaction was incubated for 60 min at 37° C.

4.5. Biochemical characterization of DprA role in genetic recombination of *B. subtilis*

DprA is a ubiquitous protein present even in bacteria lacking chromosomal transformation. In fact, *E. coli* cells have two DprA orthologs ($DprA_{Eco}$ and Smf_{Eco}) (reviewed in Kidane, Ayora et al. 2012). *In vivo* analyses revealed that DprA interacts with SsbB, RecA, and with itself (Kramer, Hahn et al. 2007). $DprA_{Spn}$ also interacts with $RecA_{Spn}$ (Mortier-Barriere, Velten et al. 2007). It has recently been shown that $DprA_{Spn}$, which crystallizes as a dimer, physically interacts with $RecA_{Spn}$ (Quevillon-Cheruel, Campo et al. 2012). *In vitro* $DprA_{Spn}$ binds ssDNA, loads the heterologous $RecA_{Eco}$ onto ssDNA (even ssDNA coated by the heterologous SSB_{Eco} protein), and it forms a mixed $DprA_{Spn}$·ssDNA·$RecA_{Eco}$ nucleoprotein filament that is proficient for DNA strand exchange (Mortier-Barriere, Velten et al. 2007). It has been proposed that $DprA_{Spn}$ might interact with naked ssDNA as soon as it exits from the entry channel, and then the $DprA_{Spn}$·ssDNA complex facilitates $RecA_{Spn}$ loading onto ssDNA (Mortier-Barriere, Velten et al. 2007).

To gain further insight into which nucleation effecter is used to load RecA onto ssDNA during chromosomal transformation and in plasmid establishment, the role of DprA was investigated. RecA, in a dATP bound form, is essential for RecA nucleation onto ssDNA, and dATP hydrolysis makes RecA·ssDNA filaments dynamic. Both steps are crucial for a successful GR reaction (Kidane, Carrasco et al. 2009, Yadav, Carrasco et al. 2012)

4.5.1. Biochemical characterization of DprA·ssDNA complexes by AFM

To analyze the mechanism by which ssDNA interacts with DprA, increasing concentrations of the protein were incubated with ssDNA, and the complexes were visualized by AFM. DprA·ssDNA complex formation was detected at DprA concentrations as low as 0.1 nM (KD_{app} ~0.3 nM), but formation of DprA·dsDNA complexes was not observed even at DprA concentrations as high as 20 nM (data not shown).

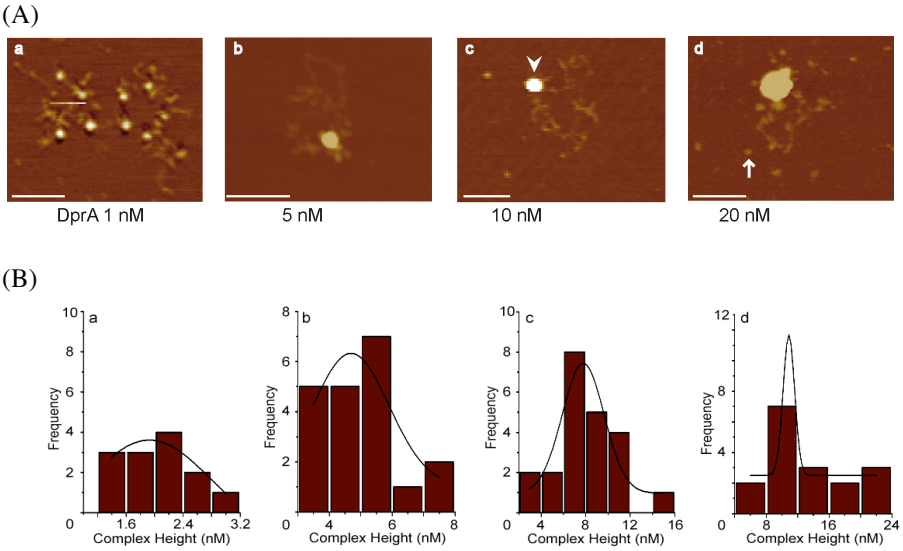

Figure 25. Binding analysis of DprA protein: (A) Binding of DprA to 3,199-nt pGEM3 Zf(+) ssDNA. pGEM3 Zf(+) ssDNA (0.1 nM in ssDNA molecules) was incubated with increasing concentrations of DprA (1, 5, 10 or 20 nM) in Buffer I containing 50 µM spermidine for 10 min at 37°C. An arrow indicates the size of free DprA. **(B)** Histogram for ssDNA·protein complexes. DprA·ssDNA complexes at 1, 5, 10, and 20 nM of DprA (a – d). Origin6 software was used to draw histograms and Gaussian curves.

At low ratios (1 DprA/ 320-nt), DprA formed protein·ssDNA complexes with an average of ~4 ± 1 DrpA per ssDNA molecule (Figure 25), but at higher protein:ssDNA ratios, DprA formed discrete globular shaped structures (termed blobs) on ssDNA that were larger than expected for dimers. At ratios of 1 DprA/ 64- to 16-nt, DprA formed large blobs on ssDNA with an average of 1.2 DprA complexes per ssDNA molecule (Figure 25A), implying that DrpA bound to ssDNA interacted with other protein·ssDNA complexes to form a discrete higher-order DprA·ssDNA complex.

The DprA structures were globular in shape, and their height and width increased with increasing protein concentrations. The theoretical volume of DprA was ~54 nm^3 for a monomer and ~108 nm^3 for a dimer. The volume of free DprA, which did not vary with protein concentration under these experimental conditions, correlated with DprA monomers (Figure 25A, c and d). The volume of DprA·ssDNA complexes, however, varied with protein concentration. At low DprA concentrations, two discrete DprA subpopulations with volumes of ~80 and ~140 nm^3, that might correlate with DprA monomers and dimers, were observed (Figure 25). At high protein concentrations, large DprA aggregates bound to one or more ssDNA molecules were observed with a volume increase of 10- to 60-fold (Figure 25B). It is likely that DprA binds with itself in a cooperative manner.

4.5.1.1. DprA promotes limited SSB dislodging from ssDNA

To gain an insight on the mechanism by which DprA interacts with ssDNA pre-coated by SsbA or SsbB, AFM experiments were performed.

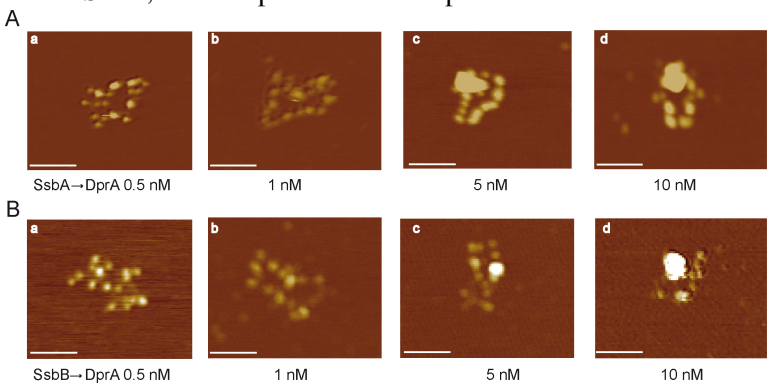

Figure 26. Binding of SSBs and DprA to 3,199-nt pGEM3 Zf(+) ssDNA. ssDNA was pre-incubated with 5 nM SsbA (**A**) or SsbB (**B**), for 5 min in Buffer I, then increasing amounts of DprA (1, 5 or 10 nM) were added and the reaction was further incubated for 5 min at 37°C in a 20 µl volume in Buffer I containing 50 µM spermidine. A fraction of the sample was deposited onto freshly cleaved mica and processed as previously described (Manfredi, Suzuki et al. 2010).

ssDNA was pre-incubated with a SSB protein at ratios of 1 SSB/ 64-nt, and then variable amounts of DprA were added (Figures 26A and 26B). At stoichiometric amounts of DprA relative to SSB, SsbA or SsbB beaded complexes were similar to those formed in the absence of DprA (Figure 26). Under this experimental condition, the height and width of DprA was similar to the beads of the SsbA and SsbB proteins, although the average number of beads (or blobs) per ssDNA molecule was reduced to 15 ± 3 and 13 ± 2, respectively (Figure 26A and 26B). The DprA blobs increased in size with increasing protein concentrations. We asked whether, at high DprA:ssDNA ratios (1 DprA/ ~64- to 32-nt), the DprA blobs, which were clearly distinguishable from the SSB beads, might dislodge the SSB proteins from the ssDNA. The number of SsbA or SsbB beads was reduced to ~13 ± 1 and ~10 ± 2 beads per ssDNA molecule, respectively (Figure 26A and 26B). Similar results were observed when stoichiometric amounts of SsbA and SsbB (1 SSB/ 32-nt) were co-assembled onto ssDNA and then DprA (1 DprA/ ~64-nt) was added. In the presence of high DprA:ssDNA ratios, in addition to DprA blobs there were only 12 to 10 SSB beads (independent of the order of addition) per ssDNA molecule (data not shown). Spontaneous dislodging of about half of the SSB beads in the 10 min reaction was unanticipated, because the half-lives for both SsbA·ssDNA and SsbB·ssDNA complexes were longer than 25 min, even for short ssDNA segments (e.g., dT_{80}) (Figure 11C), and we established that the half-life of the DprA·ssDNA was also long-lived (more than 25 min) (data not shown). This contrasts with our prior assumption that the SSB proteins are transient intermediates that do not compete with DprA for access to ssDNA, and the interaction of DprA with a SSB protein might reduce the diffusion rate of the latter. A similar destabilizing role was previously proposed for $RecO_{Eco}$ upon interacting with SSB_{Eco} (Ha, Kozlov et al. 2012), and we extended it to the RecO·ssDNA·SsbA complex (Yadav, Carrasco et al. 2012).

4.5.2. Biochemical characterization of DprA for chromosomal transformation

4.5.2.1. DprA does not facilitate RecA nucleation onto naked ssDNA

It is generally believed that during DNA repair SSB proteins coat ssDNA in the cell, and the normal substrate for RecA self-assembly *in vivo* is SSB-coated ssDNA (Shereda, Kozlov et al. 2008). However, in the context of GR this assumption was recently challenged. It has been proposed that during GR, $DprA_{Spn}$ interacts with the internalised ssDNA as soon as it

exits the entry channel without a need for SSB_{Spn} protein displacement (Mortier-Barriere, Velten et al. 2007). To test whether DprA facilitated nucleation and/or RecA filament extension onto naked ssDNA, the rate of dATP hydrolysis was determined.

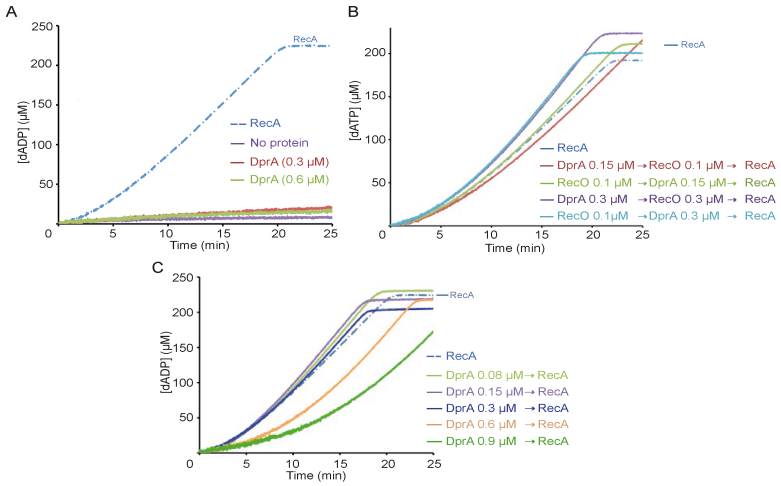

Figure 27. DprA plays a role in the rate-limiting nucleation of RecA. (A) DprA controls, (B) Effect of RecO and DprA for RecA growth. (C) The 3,199-nt pGEM3 Zf(+) ssDNA (10 μM in nt) was pre-incubated with DprA (80, 150, 300, 600 or 900 nM) in Buffer G containing 5 mM dATP. Then RecA (800 nM) was added and the absorption was monitored for 25 min.

In control experiments in the absence of RecA, DprA (1 DprA /11-nt) did not exhibit dATP hydrolysis activity (Figure 27A), indicating that the hydrolysis of dATP observed in the assays can be solely attributed to the RecA protein. At lower ratios (1 DprA/ 125- to 66-nt), DprA bound to ssDNA altered neither the lag period of RecA nucleation nor RecA·ssDNA filament formation when compared with RecA alone (Figure 27C). However, at about equimolar concentrations with RecA (1 DprA /16- to 11-nt), DprA delayed RecA nucleation onto a naked ssDNA (7 - 8 min and 12 - 13 min, respectively) relative to the lag time of RecA alone (< 5 min), and reduced the RecA filament extension phase (Figure 27C). This apparent paradox is also evident in studies of BRCA2, where high concentrations of the BRC peptide inhibit Rad51·ssDNA filament formation, while lower concentrations can stabilize the filament (Galkin, Esashi et al. 2005, Holthausen, van Loenhout et al. 2011).

To test whether the presence of a second mediator (e.g., RecO) facilitates DprA-mediated recruitment of RecA onto naked ssDNA, DprA or RecO were pre-incubated with ssDNA, the second mediator was added (RecO or DprA), and then RecA nucleation was monitored. RecA (1 RecA monomer /12-nt) added to DprA (1 DprA /66-nt) bound to ssDNA, or to the DprA·ssDNA·RecO complex resulted in a lag phase similar to that in the absence of any mediator, and it was independent of their (DprA or RecO) order of addition (Figure 27B). To determine the extent to which a mediator was affecting the slow step in the nucleation of RecA filaments, a higher DprA concentration was used (1 DprA /33-nt). The circular ssDNA was pre-incubated with DprA, and then RecO and RecA were added. Independent of the order of addition of the mediators, the lag in dATP hydrolysis was not reduced, but the rate of RecA-mediated dATP hydrolysis was significantly increased when compared to RecA alone. It is likely that neither RecO nor DprA accelerated RecA nucleation onto naked ssDNA (Figure 27B). At present we cannot discriminate whether DprA- and RecO-bound to ssDNA contribute to the spontaneous destabilization of DNA secondary structures on ssDNA, or if both proteins, by physical interaction with RecA, might facilitate the dynamic growth of the RecA·ssDNA filament.

4.5.2.2. DprA and SsbB interaction facilitates RecA nucleation onto ssDNA

In vivo the template for RecA nucleation during DNA repair is SsbA-coated ssDNA (Carrasco, Manfredi et al. 2008, Manfredi, Carrasco et al. 2008), and DprA physically interacts with SsbB and RecA (Kramer, Hahn et al. 2007); thus, we hypothesized that DprA might stimulate RecA nucleation onto SsbB-coated ssDNA during GR. To test this hypothesis, the kinetics of DprA-mediated nucleation of RecA onto SsbB- or SsbA-coated ssDNA were analysed by measuring RecA-mediated hydrolysis of dATP. In control experiments in the absence of RecA, none of the SSB protein exhibited dATP hydrolysis activity (Figure 14A).

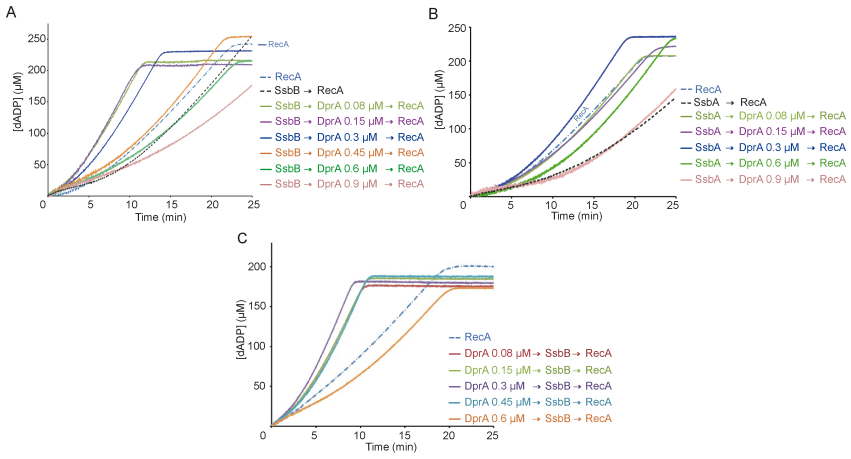

Figure 28. SsbA, SsbB and DprA plays role in the rate-limiting nucleation of RecA. The 3,199-nt ssDNA was pre-incubated with 300 nM SsbB (**A**) or SsbA (**B**), and incubated with DprA (80, 150, 300,600 or 900 nM) in Buffer G containing 5 mM dATP Then RecA (800 nM) was added and the ssDNA-dependent dATPase activity was measured for 25 min. The amount of dADP was calculated as described (Arenson, Tsodikov et al. 1999). (C) DprA added before SsbB and then RecA dATPase activity was measured for 25 min.

Pre-incubation of ssDNA with SsbA or SsbB, sufficient to saturate the binding sites (1 SSB/33-nt) prolonged the nucleation of RecA to ~11 and ~7 min, respectively, and reduced the RecA filament extension phase, albeit to a different extent (Figures 14B, 28A and 28B) (Yadav, Carrasco et al. 2012).

The effect of DprA addition on RecA nucleation and RecA·ssDNA filament extension onto SsbA-coated ssDNA was measured. At low ratios (1 DprA / 33- to 125-nt), DrpA did not significantly reduce the lag time of RecA nucleation onto SsbA-coated ssDNA, but it reversed the inhibitory effect exerted by the SsbA protein on RecA-mediated dATP hydrolysis, when compared to RecA nucleation onto naked ssDNA (Figure 28B). By contrast, DprA (1 DprA / 33- to 125-nt) significantly reduced the lag time and accelerated RecA nucleation onto SsbB-coated ssDNA (Figure 28A). DprA markedly increased RecA·ssDNA filament extension onto SsbB-coated ssDNA when compared to RecA nucleation onto naked ssDNA (Figure 28A). Similar results were seen when SsbB was added after DprA, but prior to RecA (Figure 28C). It is likely; therefore, that DprA, upon interaction with SsbB, might facilitate new nucleation at additional sites, and thereby indirectly accelerate the dynamic assembly and disassembly of RecA from ssDNA with subsequent hydrolysis of dATP (Figure 28A).

A different outcome was observed at higher DprA:ssDNA ratios. At a ratio of 1 DprA / 16-nt, DprA partially overcame the inhibitory effect exerted by SsbA (Figure 28B) or SsbB (Figure 28A) on the loading of RecA onto SsbA- or SsbB-coated ssDNA, but at a ratio of 1 DprA / 11-nt, DprA did not overcome the inhibitory effect exerted by SsbA or SsbB on RecA loading onto SsbA- or SsbB-coated ssDNA (Fig 28A and 28B). However, when ssDNA concentrations were increased, the inhibitory effects exerted by DprA on RecA nucleation and filament extension were overridden, suggesting that DprA, free in solution, interacts with

RecA to form an inactive binary, rather than inhibition of dATP hydrolysis by traces of a putative "*inhibitory factor*" present in the DprA preparation (data not shown).

4.5.2.3. DprA and SsbB interaction facilitates RecA nucleation onto SsbA-coated ssDNA

In the previous section it was shown that DprA facilitates RecA nucleation and filament extension onto SsbB-coated ssDNA (Figures 28A and 28C), but merely counters the inhibitory effect exerted by SsbA on RecA nucleoprotein filament formation (Figure 28B). To test whether DprA, upon interacting with SsbB bound to ssDNA, licensed the dislodging of both SsbA and SsbB co-assembled onto ssDNA, the rate of dATP hydrolysis was determined. SsbA and SsbB co-assembled onto ssDNA increased the lag time of RecA nucleation to levels comparable to the first SSB protein added (Figure 29). To test whether the order of SSB protein assembly affected the outcome, SsbA was pre-incubated with ssDNA, and SsbB was added (or *vice versa*), followed by addition of DprA, then RecA was added and finally RecA-mediated dATP hydrolysis was measured.

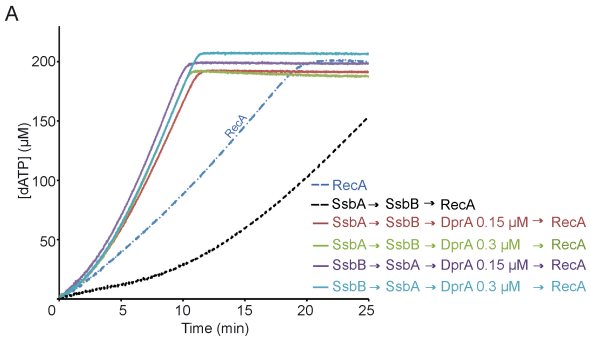

Figure 29. SsbA, SsbB and DprA plays role in the rate-limiting nucleation of RecA. DprA facilitates RecA loading onto SsbA·ssDNA·SsbB. (**A**) The 3,199-nt pGEM3 Zf(+) ssDNA (10 μM in nt) was pre-incubated with SsbA, SsbB, or with SsbA and then with SsbB (SsbA → SsbB) or *vice versa* (SsbB → SsbA) in Buffer G containing 5 mM dATP. DprA (150 nM or 300 nM) was added and incubated for 5 min. RecA was then added and the absorption measured for 25 min.

Addition of DprA (1 DprA/ 33- to 66-nt) to the SsbA·ssDNA·SsbB complex markedly shortened the rate-limiting RecA nucleation time to <2 min and facilitated dATP hydrolysis (RecA polymerization) independently of the order of addition (Figure 29). Similar results were observed if the SSB protein concentration was reduced by half (data not shown). Since the second SSB protein was added after the first was already in a complex with ssDNA, formation of heterotetrameric proteins was unlikely.

To test whether DprA bound to ssDNA constrained the diffusion of the SSB protein along the ssDNA lattice and facilitated its spontaneous dislodging, the steady state rate of RecA-mediated dATP hydrolysis was measured in the presence of a heterologous SSB protein (Ssb_{SPP1}).

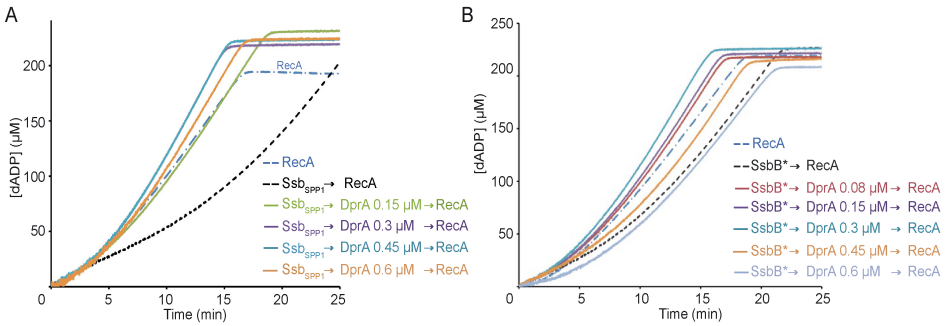

Figure 30. DprA overcomes the interference of a heterologous SSB or SsbB* protein on RecA nucleation. (A) The 3,199-nt ssDNA (10 μM in nt) was pre-incubated with 300 nM Ssb_{SPP1} and then incubated with DprA (150, 300,600, or 900 nM). Then RecA (800 nM) was added, and the ssDNA-dependent dATPase activity measured for 25 min. (B) ssDNA (10 μM in nt) was pre-incubated with 300 nM SsbB* and then incubated with DprA (150, 300, 450, or 600 nM).

Pre-incubation of ssDNA with Ssb_{SPP1} sufficient to saturate the binding sites (1 tetramer /33-nt) retarded the nucleation of RecA onto ssDNA, with a lag phase of 7 - 8 min, and also reduced the RecA filament extension (Figure 30A). The presence of DprA did not significantly decrease the nucleation time of the RecA protein onto Ssb_{SPP1}-coated ssDNA, but it reversed the inhibitory effect exerted by Ssb_{SPP1} on the extension of RecA filaments (Figure 30A), suggesting that DprA might facilitate partial displacement of a heterologous SSB protein. This is consistent with the observation that $DprA_{Spn}$ partially displaces SSB_{Eco} from ssDNA (Mortier-Barriere, Velten et al. 2007).

The acidic C-terminal tail of SSB proteins is crucial for interactions with many proteins (Shereda, Kozlov et al. 2008). Recently it was proposed that the acidic C-terminal tail of $SsbB_{Spn}$ is involved in the specific interaction with other processing proteins(s), such as RecA or DprA (Attaiech, Olivier et al. 2011). In the previous section it was shown that DprA facilitates RecA nucleation onto SsbB-coated ssDNA (Figure 28A). To determine whether a chimeric acidic C-terminal end added onto SsbB might play any role in the interaction with DprA, and/or in the recruitment of RecA, SsbB* was used. And also to test whether SsbB* acts in the recruitment of RecA by DprA, the rate of dATP hydrolysis was determined. Similar to all SSB proteins, SsbB* interfered with RecA nucleation onto naked ssDNA (Figure 30B). At lower ratios (1 DprA /125- to 33-nt), DprA overcame the inhibitory effect on RecA nucleation onto SsbB*-coated ssDNA, but it did not significantly contribute to reduce the lag time of RecA nucleation onto SsbB*-coated ssDNA (Figure 30B). It is likely that the presence of the acidic C-terminal tail produces a subtle change in the SsbB moieties, so that DprA could not effectively interact with it. At high DprA:ssDNA ratios, DprA also inhibited the nucleation of RecA onto SsbB*-coated ssDNA (Figure 30B).

4.5.2.4. DprA facilitates RecA-mediated DNA strand exchange

To understand the effect of SsbA, SsbB, and DprA on RecA activities, RecA-mediated DNA strand exchange was evaluated using a three-strand exchange reaction in the presence of dATP as a nucleotide cofactor.

In the absence of SsbA or SsbB, RecA catalysed dATP-dependent strand exchange between circular ssDNA (*css*) and a linear dsDNA (*lds*), converting ~ 18% of the homologous ldsDNA into joint molecules (*jm*) and final nicked-circular (*nc*) products during a 60 min reaction (Figure 31A and 31B, lane 2). The addition of half-saturating to saturating SsbA or SsbB (1 tetramer/ 66-, 33- or 22-nt) significantly stimulated RecA strand exchange (~ 4- and ~ 3-fold, respectively) as judged by the accumulation of dATP-dependent *jm* intermediates and *nc* products (Figure 31A, lanes 3-5 and 6-8). It is likely that the SSB protein aids RecA-mediated DNA strand exchange by facilitating spontaneous melting of DNA secondary structure and by sequestering the displaced strand, which is inhibitory to the RecA-mediated DNA strand exchange reaction.

The addition of DprA (1 tetramer per 66-, 33- or 22-nt) also stimulated RecA-mediated DNA strand exchange (~3-fold) (Figure 31A, lanes 9-11). To test whether DprA acted by recruiting RecA onto SsbB- or SsbA-coated ssDNA, RecA-mediated strand exchange in the presence of a constant amount of a SSB protein (1 SSB/66-nt) and increasing DprA concentrations was analysed. The accumulation of *jm* intermediates and *nc* product was increased, compared with RecA alone (Figure 31A, lanes 2). In the presence of SsbB, the DprA concentration could be lowered 3-fold without affecting the optimal efficiency of RecA-mediated DNA strand exchange (Figure 31A, lanes 11 and 12). The presence of DprA and SsbB facilitated the formation of dynamic RecA filaments, but resulted in an apparent inefficient strand exchange reaction when compared to the presence of DprA and SsbA (Figure 31A, lanes 12-17). Since nucleotide binding, but not hydrolysis, leads to the formation recombination products (Forget and Kowalczykowski 2012), we can argue that a protein(s) that stabilises the RecA·ssDNA filament and/or leads to an effectual lengthening of the RecA·ssDNA filament is missing in the strand exchange reaction in the presence of DprA and SsbB (Bell, Plank et al. 2012, Cardenas, Carrasco et al. 2012).

RecA interacts with DprA, SsbB, and with itself (Kramer, Hahn et al. 2007). To test whether DprA could facilitate RecA-mediated DNA strand exchange between ssDNA coated by a SSB protein, the ssDNA was pre-incubated with variable concentrations of both SSB proteins (1 tetramer/ 66-, 33- or 22-nt).

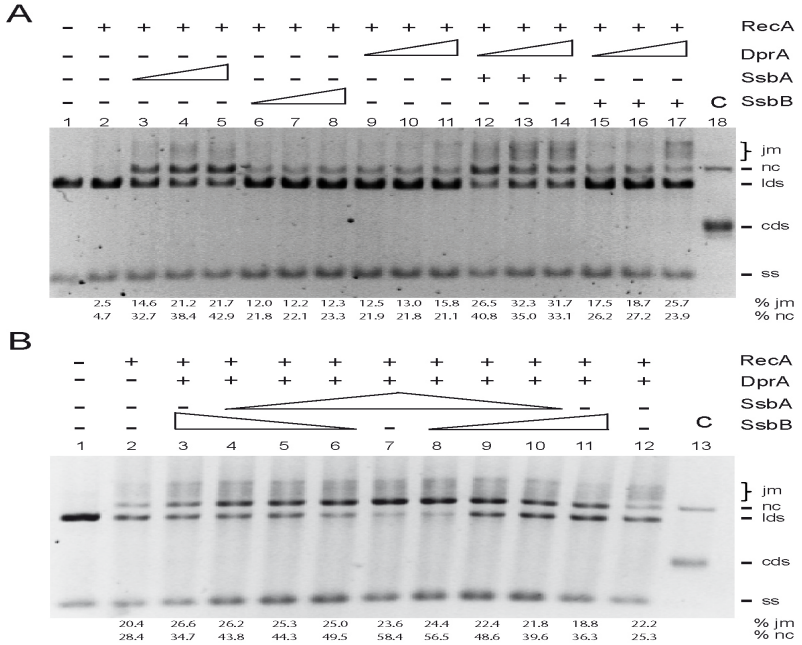

Figure 31. DprA facilitates RecA-mediated DNA strand exchange in the presence of both SSB proteins. (**A**) Circular 3,199-nt pGEM3 Zf(+) ssDNA (10 μM in nt) and homologous KpnI-linearised dsDNA (20 μM in nt) were pre-incubated with increasing concentrations of SsbA [150, 300 and 450 nM (lanes 3-5)], SsbB [150, 300, and 450 nM (lanes 6-8)], and DprA [150, 300, and 450 nM (lanes 9-11)] for 10 min at 37 °C in Buffer G containing 2 mM dATP. The circular ssDNA and homologous KpnI-linearised dsDNA were pre-incubated with a constant amount of SsbA (150 nM in lanes 12 to 14) or SsbB (150 nM in lanes 15 to 17). Then increasing concentrations of DprA (150, 300 and 450 nM) were added and incubated for 10 min at 37° C. Then a constant amount of RecA (700 nM, lanes 2-17) was added and the reaction was incubated for 60 min at 37° C. (**B**) Circular ssDNA and homologous linear dsDNA were pre-incubated with decreasing concentrations of SsbB and then increasing concentrations of SsbA (lanes 3-7) or *vice versa* (lanes 7-11) for 5 min at 37° C in Buffer G containing 2 mM dATP. The complex was incubated with a constant amount of DprA (100 nM, lanes 3-12) for 5 min at 37° C, followed by addition of a constant amount of RecA (700 nM, lanes 2-12) and incubated for 60 min at 37° C. The products of the reactions were deproteinised and separated on a 0.8% agarose gel with ethidium bromide. The position of the bands corresponding to *css*, *lds*, *nc*, *jm* and *ccc* are indicated. + or - denote the presence or absence of the indicated protein.

SSB-bound ssDNA was incubated with limiting DprA concentrations (1 DprA/ 100-nt) followed by addition of RecA (1 RecA/ 14-nt) bound to dATP. In the presence of the preformed SsbA·ssDNA·SsbB complex, addition of DprA increased RecA-mediated DNA strand exchange by ~ 4-fold (Figure 31B, lane 6-9). It is likely that the interaction of DprA with SsbB in the SsbA·ssDNA·SsbB complex facilitated RecA-mediated DNA strand exchange when compared with RecA alone or DprA and RecA (Figure 31B, lanes 2 and 12).

4.5.3. Biochemical characterization of DprA for plasmid transformation

4.5.3.1. DprA facilitates annealing of naked or SSB-coated DNA strands

In a previous section it was shown that: i) SsbA or SsbB bound to ssDNA failed to bridge two non-complementary DNA molecules by a direct protein-protein interaction (Figure 12A and 12B); ii) DprA on one ssDNA molecule interacted with DprA·ssDNA on a second ssDNA molecule and bridged them (Figure 25A); and iii) DprA is necessary for effective plasmid transformation (Table 7). However, it was reported that DprA was unable to catalyse ssDNA annealing under conditions where $DprA_{Spn}$ does (quoted in Claverys, Martin

et al. 2009). To test whether DprA is able to catalyse DNA strand annealing, heat-denatured 440-nt long complementary ssDNA strands coated or not by a SSB protein were examined.

In the presence or absence of Mg^{2+}, spontaneous annealing of the complementary heat-denatured 400-nt DNA strands was measured to be ~16% (data not shown) and ~20% (Figure 32), respectively (Manfredi, Suzuki et al. 2010, Yadav, Carrasco et al. 2012), and the presence of the SSB proteins (1 SSB/53-nt) prevented spontaneous reannealing (Figure 32B lane 10, 32C lane 4 and 32D, lane 11).

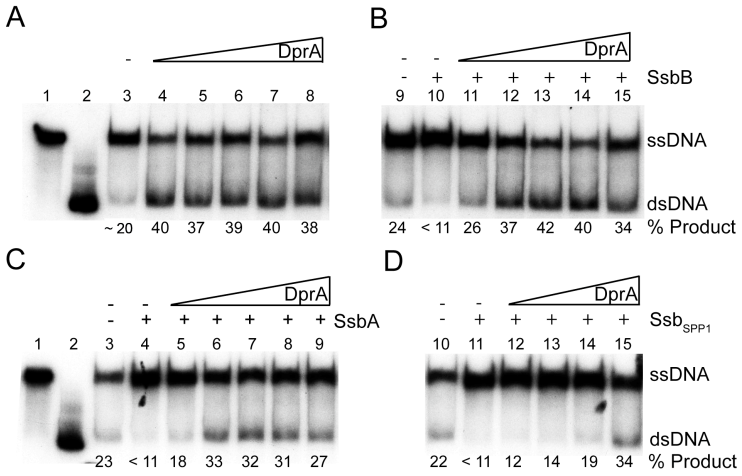

Figure 32. DprA facilitates the spontaneous annealing of naked or SSB-coated complementary DNA strands. Heat-denatured 440-nt long [g-^{32}P]-ssDNA (8 mM in nt) was quickly cooled and incubated with increasing DprA (50, 100, 200, 400 or 800 nM) concentrations (A) or pre-incubated with a fixed amount (150 nM) of SsbB (B), SsbA (C), or Ssb_{SPP1} (D) for 10 min at 30º C in Buffer H, and then incubated with increasing concentrations of DprA (50, 100, 200, 400 and 800 nM) for 60 min at 30º C. Heat-denatured ssDNA (lane 1) was slowly cooled to allow spontaneous annealing (lane 2). Note that under these electrophoresis conditions, dsDNA runs ahead of ssDNA. The products of the reactions were deproteinised, separated by 6% PAGE and monitored with a PhosphoImager (BioRad) system

At low ratios (1 DprA /160-nt), DprA lead to ~ 40% annealing of naked complementary strands (Figure 32A, lane 4), but increasing DprA concentration (1 DprA/ 80- to 10-nt) did not further enhance the SSA reaction (Figure 32A, lane 5-8). At a ratio of 1 DprA/ 80-nt, DprA-mediated annealing of the two complementary ssDNAs coated by SsbB (1 SsbB/53-nt), and at a ratio of 1 DprA/ 40-nt reannealing reached a maximal level (Figure 32B, lanes 12-13). At higher ratios (1 DprA/10-nt) a slight reduction of the SSA reaction was observed (Figure 32B, lane 15). When SsbB was replaced by SsbA, ~ 30% of the complementary strands were re-annealed at ratios of 1 DprA/ 80-nt (Figure 32C lanes 6-8). By contrast, DprA protein annealed Ssb_{SPP1}-coated ssDNA complexes very poorly, and higher protein/ssDNA ratios (1 DprA/ 20-nt) were needed (Figure 32D, lane 15). It is likely that DprA, by interacting with SsbB, mediates annealing of complementary ssDNA tracts coated by SsbB. Moreover, and ii) DprA·DprA interactions bridging two ssDNA molecules preferentially facilitates the annealing of complementary ssDNA tracts coated by SsbB.

4.5.3.2. DprA facilitates the annealing of SsbB-coated plasmid DNA strands

To test whether DprA could mediate SSA of circular plasmid-size ssDNA substrates (pGEM3 Zf[+] and pGEM3 Zf[-]) coated or not by a SSB protein, the strand annealing reaction was performed and then monitored by AFM.

When increasing concentrations of DrpA (1 DprA/ 500- to 125-nt) were incubated with both ssDNA molecules, intermolecular bridging was apparent (Figure 33C, frames a to c), but accumulation of duplex DNA was seldom observed (<1%). Similarly, when SsbA (1 SsbA/ 64-nt) was pre-incubated with the ssDNAs and then increasing concentrations of DprA (1 DprA/ 500- to 125-nt) were added, DprA-mediated bridging and accumulation of duplex DNA were rarely observed (Fig 33A).

Importantly, when the ssDNA was pre-coated with SsbB, DprA (1 DprA/ 250-nt) annealed plasmid-size complementary ssDNA (Fig 33B), suggesting that the annealing of plasmid-sized ssDNA strands is preferentially dependent on SsbB. As revealed in Figure 33B, the annealed duplex is largely devoid of associated proteins, supporting the conclusion that DprA showed lower affinity for duplex DNA when compared with ssDNA.

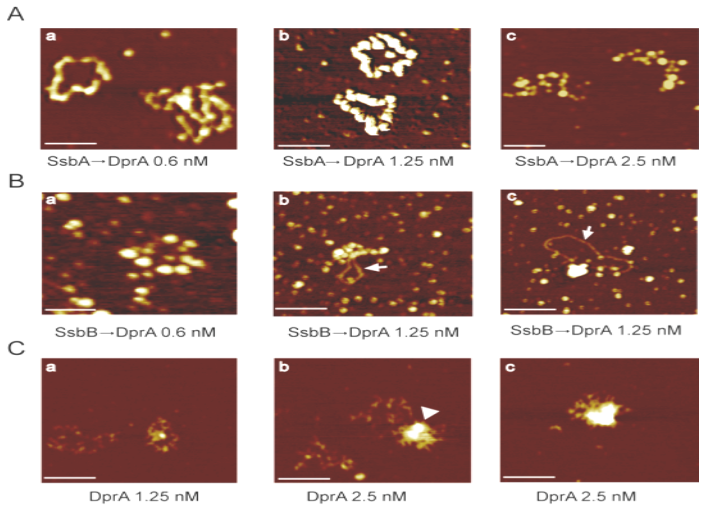

Figure 33. DprA-mediated strand annealing analysis by AFM in the absence or presence of SsbA, and SsbB. 3,199-nt pGEM3 Zf(+) and pGEM3 Zf(-) ssDNA (0.1 nM each in ssDNA molecules) were pre-incubated with SsbA (5.0 nM) (A) or SsbB (5.0 nM) (B), and then incubated with increasing DprA concentrations (0.6, 1.25 and 2.5 nM) in Buffer I containing 50 μM spermidine for 30 min at 30˚C in a 20 μl reaction mixture. (C) The complementary 3,199-nt pGEM3 Zf(+) and pGEM3 Zf(-) ssDNA (0.1 nM each in ssDNA molecules) were incubated with DprA (1.25 and 2.5 nM) in Buffer I containing 50 μM spermidine for 30 min at 30˚C in a 20 μl reaction mixture. A fraction of the sample was deposited onto freshly cleaved mica and processed as previously describe.

In the presence of large DprA concentrations (1 DprA/ 1.7-nt and 0.2-nt in the absence and presence of Mg^{2+}, respectively) DprA·dsDNA complexes that did not enter polyacrylamide gels were observed (data not shown). It is likely that DprA binds dsDNA in a sequence-independent manner. Similarly, $DprA_{Spn}$ shows no detectable affinity for dsDNA although it shows a significant binding to supercoiled circular DNA (Mortier-Barriere, Velten et al. 2007).

5. DISCUSSION

5. Discussion

Four competence-induced proteins are known to be required for the early stages of transforming DNA in *B. subtilis*: RecA, SsbA, SsbB, and DprA (Berka, Hahn et al. 2002, Hamoen, Smits et al. 2002, Ogura, Yamaguchi et al. 2002), and a fifth protein (CoiA), with unknown activity, might work at late stages. These proteins, except SsbA, are also induced in *S. pneumoniae* competent cells (Claverys, Martin et al. 2009). The universal DNA strand-exchange protein, RecA, is essential for chromosomal transformation in all described competent cells (Claverys, Martin et al. 2009, Kidane, Ayora et al. 2012). RecA bound to dATP is able to promote DNA strand exchange to form hybrid DNA *in vitro* without additional proteins. However, accessory factors can stimulate the strand exchange reaction. These factors can be divided into two broad classes: those that act **before homology search** by promoting assembly of RecA·ssDNA filaments, and those that act **during homology search** by modulating the length of the RecA·ssDNA filaments (Alonso, Cardenas et al. 2013). Assembly factors can, in turn, be divided into two classes: the SSB protein (SsbA and SsbB) and the "mediators" (RecO and DprA). Here we have focused on the roles of assembly factors that act before homology search.

The SsbA and SsbB proteins share 63% identity at N-terminal domains (residues 1–106), which is responsible for ssDNA binding and subunit tetramerization. SsbA and SsbB proteins are different in their C-terminal moiety. The SsbA C-terminal contains a characteristic acidic tail, which is responsible for protein-protein interaction in several bacteria (Costes, Lecointe et al. 2010), whereas SsbB lacks this acidic and unstructured tail. Biochemical study have been done for SsbA and SsbB binding, and it suggests that both proteins have two modes for binding with ssDNA, correlating to the SSB_{65} and SSB_{35} binding modes (Figures 8,-10). These modes of binding of SsbA and SsbB are conserved in other bacterial phyla. The crystal structure studies have been done for SsbB·ssDNA co-complex (Yadav, Carrasco et al. 2012) and it shows that protein forms tetramer as functional unit. The SsbB protein has similar ssDNA binding surfaces in its tetramer form. However, SsbB structure reveals that there is a significant difference in structure from other SSBs. The SsbB appears to interact with ssDNA in a manner that 8-10 bases of ssDNA faces toward the protein core (Yadav, Carrasco et al. 2012) rather than the corresponding bases point towards the solvent as the SSB_{Eco} complexed with ssDNA (Raghunathan, Kozlov et al. 2000).

Biochemical study showed that the SsbA protein has > 10-fold higher binding affinity for ssDNA than the SsbB protein (Figures 8-10, Table 5). However in case of *S. pneumoniae* the two SSBs bind ssDNA with opposite affinity, where $SsbA_{Spn}$ binds with less affinity than the $SsbB_{Spn}$ (Grove and Bryant 2006, Salerno, Anne et al. 2011). This difference in binding affinities may be attributed to the difference in their C-terminal domains (Costes, Lecointe et al. 2010). To examine the role of the acidic tail of SSB proteins, we designed a chimeric protein, which contains full-length SsbB and the nine-most C-terminal residues of SsbA, the variant was termed as SsbB*. This protein shows increased ssDNA-binding affinity (Table 6), but it fails to interact with the RecO mediator, suggesting that the extreme C-terminal end of SsbA is not the only region of SsbA which is responsible for interaction with RecO.

AFM studies have been done to understand the nature of the SSB·ssDNA (Figure 12), and SsbA·ssDNA·RecO complexes (see Manfredi, Suzuki et al. 2010). The circular ssDNA was visualized as compact collapsed thread structure, which was changed in to beaded structure after addition of SsbA or SsbB (Figure 12). The morphologies of the bound SsbA·ssDNA and SsbB·ssDNA complex were similar among them (Figure 12), and related to those observed for tetrameric SSB_{Eco} in the presence or absence of Mg^{2+} (Hamon, Pastre et al. 2007, Li and Goh 2010). These suggest that the binding natures of both SSBs with ssDNA are similar.

Binding nature of RecO protein has been observed by biochemical and biophysical (AFM) assays. The N-terminal domain of RecO has the ssDNA-binding domain. RecO binds with ssDNA as discrete globular shaped structures, which is larger than the monomer volume.

This is consistent with the observation that RecO forms dimers in protein cross-linking experiments (Manfredi, Carrasco et al. 2008).
The DprA binding nature has been examined by AFM and biochemically. The study revealed that at low concentrations, DprA forms many discrete blobs onto naked ssDNA, whereas at higher DprA:ssDNA ratios only one discrete and large globular shaped structure per DNA molecule have been observed. This nature of binding of DprA as a single structure per ssDNA molecule, at high protein:DNA ratio, does not support the hypothesis that DprA protects the ssDNA from degradation (see Bergé, Mortier-Barriere et al. 2003). The volume of DprA, at the protein·ssDNA complexes, increased with increasing DprA concentrations, which can be attributed to high self-interaction affinity for DprA protein than the ssDNA. The self-interaction of DprA might lead to form a large high-order $DprA_n$·ssDNA complex (Figure 25), and also bridging ssDNA·$DprA_n$·ssDNA complex where two ssDNA molecules interact via DprA. Alternatively, the different DprA·ssDNA complexes are short-lived and by cooperative protein·protein interactions a discrete DprA·ssDNA complex was formed. Our studies suggest that DprA binding to ssDNA was different than the one of SSB proteins. A DprA discrete protein·ssDNA complex was bound, and two DprA blobs could interact to form bridging complexes, hence coating of the ssDNA was not observed. These suggest us that the mode of interaction of DprA with ssDNA was conflicting with the hypothesis that DprA might protect the ssDNA from endo- or exonuclease degradation (Dwivedi, Sharma et al. 2013). Furthermore, binding of DprA with ssDNA as blobs of the variable size, and SSBs as beaded structure of homogeneous size were observed at the same ssDNA molecule, suggesting that both DprA and SSB can coexist at the same molecule, and an accessory protein(s) other than DprA might be needed to protect the incoming ssDNA from degradation.

5.1. Recombination mediators have specificity for SSBs in chromosomal transformation:

The rate-limiting step in RecA filament assembly is the nucleation process. Pre-bound SSB (SsbA, SsbB or SSB_{SPP1}) to ssDNA creates a significant kinetic barrier to RecA nucleation and filament extension, and negatively regulate RecA filament growth on ssDNA. This negative regulation by SSB proteins for RecA growth leads to highlight the role of RecA mediators.

It is generally believe that the SSB proteins have a dual role. SSB_{Eco}, if added before $RecA_{Eco}$, acts as barrier for RecA filament growth onto ssDNA and strand exchange, but enhances filament growth and strand exchange if added after $RecA_{Eco}$. This enhancement could be because SSB_{Eco} facilitates the spontaneous melting of DNA secondary structure (Kowalczykowski, Dixon et al. 1994). In case of *B subtilis*, SsbA protein plays essential roles in DNA replication, recombination, and repair. SsbA inhibits RecA-catalyzed ssDNA-dependent hydrolysis of dATP, independently of the order of addition. Pre-coating of ssDNA by SsbA leads to high restriction for RecA nucleation and RecA filamentation. SsbA binding onto ssDNA is important to protect incoming ssDNA by nucleases. At the same time SsbA should be displaced for RecA filament growth onto ssDNA.

RecO is a genuine RecA mediator during DNA repair, but its role during chromosomal transformation was poorly understood. RecO alone does not change rate of RecA nucleation, and filament growth onto ssDNA (Figure 14B). RecO, which physically interacts with SsbA, reverts the negative effect exerted by SsbA on RecA nucleation, and facilitates RecA growth (Figure 14). A His-tagged RecR variant, under the conditions used, neither facilitates RecA nucleation nor filament growth onto SsbA-coated ssDNA. These data suggest that RecO alone (without RecR) interacts physically with SsbA bound to ssDNA, and modulates RecA activities for nucleation and strand exchange *in vitro*. The activity of RecO resembles the efficient loading of Rad51 onto RPA-coated ssDNA by Rad52 (Manfredi, Carrasco et al. 2008).

SsbB also exerts negative effect on RecA nucleation onto ssDNA, but it has a lower negative effect when compare to SsbA. This could be explained by the lower binding efficiency of SsbB protein for ssDNA, and RecA might displace SsbB from ssDNA more

easily. RecO fails to revert the negative effect exerted by SsbB on RecA nucleation onto ssDNA (Figure 14). SsbB does not facilitate RecA-mediated three-strand exchange reaction in presence of RecO, when compare to SsbA and RecO (Figure 17). However, RecO facilitates RecA filament growth onto ssDNA coated by both SsbA and SsbB proteins, and also facilitates the efficiency of RecA-mediated joint molecule formation (Figure 15 and Figure 17). RecO was unable to revert the negative effect exerted by SsbB* on RecA filament growth onto ssDNA (Figure 16A), which shows that RecO has some other interaction domains in SsbA protein, apart from extreme C-terminal. Recently, it was shown that *Mycobacterium smegmatis* RecO used a domain other than the C- terminal tail to interact with RecO (Gupta, Ryzhikov et al. 2013).

These studies suggest that RecO facilitates RecA filament growth and RecA-mediated DNA strand exchange in the presence of SsbA, while SsbB work as accessory factor. The specific RecO·SsbA·ssDNA interactions might lead to a limit displacement of SsbA from ssDNA and facilitate RecA filament growth onto RecO·SsbA·ssDNA complexes. This specific interaction of SsbA with RecO could lead to displacement of both proteins on SsbA·ssDNA·SsbB pre-coated ssDNA, and enhances further RecA filament growth onto RecO·SsbA·ssDNA·SsbB complex (Figure 15A). Hereafter we can say that the SsbA and accessory SsbB modulates RecA activities in a RecO mediated process.

SsbB and DprA both are induced during competence stage and co-localize at the cell pole with the uptake machinery (see Introduction). SsbB has a higher binding efficiency than DprA, and SsbB will decorate the internalized ssDNA firstly, and it will exert negative effect on RecA nucleation. We have shown hereafter that the RecA mediator, DprA, specifically interacts in concert with SsbB. AFM study suggest that DprA promoted a limited dislodging of both SSBs from ssDNA (Figure 26). DprA, at low protein concentrations, does not significantly facilitate the nucleation of RecA, suggesting that DprA might not help in removal of DNA secondary structure. Whereas at higher DprA concentrations (equimolar with RecA), RecA nucleation onto ssDNA is significantly reduced and delayed (Figure 27C). At high DprA concentration, DprA by physical interaction with RecA (Kramer, Hahn et al. 2007), might sequester it, hence reducing RecA availability. The inhibitory effect of DprA at high concentration was reverted by increasing the concentration of the ssDNA substrate.

DprA, at low protein concentrations and independently of the order of addition, facilitates RecA nucleation and filamentation onto SsbB-coated ssDNA. However, if the ssDNA was pre-incubated with SsbA, DprA partially or it does not facilitate RecA loading (Figure 28B). RecA efficiently nucleates onto the DprA·SsbB·ssDNA·SsbA complexes with similar efficiency that onto DprA·SsbB·ssDNA complexes, suggesting that DprA is able to displace co-assembled SsbB and SsbA proteins from ssDNA, and facilitates very efficient RecA filament growth (Figure 28C). We could assume that SsbA and SsbB slide along the ssDNA contribute to the spontaneous destabilization of the DNA secondary structures. The interaction of DprA with SsbB might facilitate dissociation and diffusion rate of both SSB proteins, and facilitates RecA filament growth. DprA only alleviates the kinetic barrier to RecA loading onto SsbA-, Ssb_{SPP1}- or SsbB*- coated ssDNA without a significant acceleration of the rate-limiting step of RecA nucleation (Figure 28 and Figure 30), hence providing evidence for SsbB specificity.

5.2. Nucleotide factor affects RecA nucleation and filamentation

In the cell the relative availability of ATP is 100- to 500- folds higher than the dATP. Which raises the question for RecA cofactor preferences during DNA repair and GR. RecA catalyses DNA pairing, and extensive strand exchange by a mechanism that requires nucleotide binding, but does not require hydrolysis (Menetski, Bear et al. 1990). The nucleotide cofactor preference by Firmicutes RecA was analysed. RecA nucleation and filament formation is rate limiting and it shows that RecA preferentially hydrolyzes dATP than the ATP. The efficient pH for RecA filament growth is 7.5, which is similar to physiological pH of the cell. In the presence of ATP as nucleotide cofactor, RecA nucleation

and filament growth is blocked almost entirely onto SsbA- (or SsbB)-coated ssDNA. Whereas, in presence of dATP, the RecA filament growth was reduced on SSB pre-coated ssDNA. Hence it suggests that change of cofactor leads to change in RecA activity, which is completely unable to nucleate on SSBs coated ssDNA in the presence of ATP.

In the absence of RecO, RecA nucleates on the SsbA·ssDNA or SsbA·ssDNA·SsbB complexes and catalyses DNA strand exchange using circular ssDNA coated with SsbA and SsbB in the presence of dATP (Figure 17) but ATP does not support these activities under similar conditions (Figure 24). The addition of RecO completely revert negative effect exerted by SsbA, but not by SsbB (Figure 21 and 24). Hence RecO is important mediator for RecA nucleation onto SSB-coated ssDNA, in the presence of ATP as cofactor (Figure 24). In the presence of RecO, RecA effectively nucleates on the SsbA·ssDNA or SsbA·ssDNA·SsbB complexes and catalyses DNA strand exchange in the presence of dATP or ATP (Figure 17 and 24). Since the relative availability of ATP is higher than the dATP, it is likely that ATP competes for the catalytic site of RecA, and it might be more commonly hydrolysed in *recO*$^{+}$ cells. We proposed that RecO is the key player for RecA mediated events, in the ATP bound form, where RecO requirement is absolute on SSB coated ssDNA. These results lead us to assume a role of RecO in the RecFOR pathway for RecA-mediated DNA repair, in the presence of ATP, where RecO is absolutely required. RecA in the dATP bound form might be involved in AddAB (counterpart of $RecBCD_{Eco}$) pathway of double strand break repair, where RecO role is not required. Further evidence and results are required to support these hypotheses

Our biochemical data suggesthat that RecO is sufficient to recruit RecA onto SSB-coated ssDNA *in vitro*, but the recO and recR cells show a similar phenotype and RecR suppressor also supress the reoO defect (Alonso, Cardenas et al. 2013). Furthermore, as $RecO_{Msm}$ *B. subtilis* RecO has a ZnD at the C-terminal region (see Introduction). The presence of the ZnD increases $RecO_{M.sme}$ activities in presence of Zn^{++} ions (Gupta, Ryzhikov et al. 2013), but this effect was not observed with RecO (Figure 22). *B. subtilis* RecR also has a ZnD (Alonso, Cardenas et al. 2013). We proposed that the concerted action in the presence of Zn^{++} should be re-visited.

5.3. Plasmid transformation is dependent on mediators

Plasmid transformation is a RecA-independent event and it is facilitated by proteins with the potential to anneal complementary ssDNA molecules. There are several plasmid transformation models, and in all, to reconstitute a circular duplex molecule one or more strand annealing steps are required.

SsbA, SsbB or Ssb_{SPP1}, pre-bound to ssDNA, prevents the spontaneous annealing of complementary DNA strands, hence they inhibits the annealing of the complementary strands (Figure 31). It has been widely reported that a subset of 'mediators' bears potential SSA ability, and promote the annealing of complementary strands coated with a SSB protein.

RecO has SSA potential, and it increases annealing ~ 80-fold on SsbA-coated ssDNA, irrespective of the presence or absence of Mg^{2+} ions (Figure 18) (Manfredi, Suzuki et al. 2010)). SsbA facilitates RecO-mediated strand annealing through the accumulation of non-productive ternary complexes, by protein–protein and protein–ssDNA interactions (Figure 18). The physical interaction between RecO and SsbA leads to destabilize SsbA-ssDNA complex. The possible mechanism of RecO-mediated DNA strand annealing, in presence of SsbA, could be divided in several discrete steps. First, SsbA binds to ssDNA, and it recruits RecO to form a ternary complex ssDNA–SsbA–RecO. Second, this ternary complex could interact with another RecO (present at complementary ssDNA as ternary complex). Third, by RecO-RecO interactions a bridged structure is formed, RecO destabilizes SsbA–ssDNA complexes, and facilitates dislodging from ssDNA. Finally, RecO, once bound to protein free ssDNA, distorts the structure of ssDNA, prevents SsbA binding and/or liberates it from ssDNA, and bring complementary strand together for annealing (Masuda, Ito et al. 2009, Manfredi, Suzuki et al. 2010). RecO, however, is not able to promote annealing of

complementary strands if the ssDNA is pre-coated by SsbB (Figure 18), whereas, facilitate annealing of complementary strands if the ssDNA is pre-coated by both SSB proteins (SsbA and SsbB together). RecO at the bridged structures either decreases the half-life of SsbA- and SsbB-coated ssDNA or alter the structure of ssDNA to facilitate the dissociation of both SSBs from ssDNA, possibly resulted in faster net disassembly of both SsbA and SsbB. From another side, RecO fails to increase annealing on SsbB* or Ssb_{SPP1} pre-coated complementary ssDNA (Figure 18; (Manfredi, Suzuki et al. 2010)), which suggest a role for the specific interaction of RecO and SsbA.

Another recombination mediator that works preferentially in concert with SsbB was analysed. Biochemical and AFM studies were done to understand annealing process by DprA. DprA alone has a potential SSA ability, and mediate the formation of annealed product (Figure 32A). AFM results suggest that DprA facilitates formation of bridged complexes, but not fully annealed long ssDNA molecules (Figure 33C). SsbA or SsbB pre-bound to ssDNA inhibits the spontaneous annealing of complementary DNA, and reduces the accumulation of annealed product. DprA alleviates the barrier of SsbB, and anneals complementary ssDNA pre-coated by SsbB (Figure 32B). DprA shows high preference for SsbB, and this interaction might lead to destabilize SsbB-ssDNA complex, and facilitates annealing of complementary ssDNA by protein·protein interaction and bridging mechanism (Figure 32B and 33B).

DprA-mediated strand annealed products were observed when SsbA pre-coated ssDNAs albeit with lower efficiency (Figure 32C and Figure 33A). These data suggest a lesser specificity of DprA for SsbA than the SsbB protein. When ssDNA was pre-coated by Ssb_{SPP1} instead of SsbB, the efficiency of DprA-mediated strand annealing was further reduced or barely detected (Figure 32D). It is likely that there is a different requirement for both mediators (RecO and DprA) in plasmid transformation depending on the SSB protein that coats the incoming ssDNA. Indeed, the absence of RecO or DprA reduced plasmid transformation by 30- and 40-fold, respectively (Table 7), suggesting that there are two alternative strand annealing pathways operative in the *rec*$^+$ cells for plasmid transformation. Consistently, the absence of both RecO and DprA, reduces plasmid transformation >1000-fold (Table 7). The absence of RecA, however, might alter the relative availability of both SSB proteins. SsbA, which shows higher affinity for ssDNA than SsbB, might be titrated by coating the ssDNA in the Δ*recA* background. If SsbA is titrated, free SsbB bound to plasmid ssDNA might re-direct the strand annealing reaction towards DprA, and exerts a negative effect on a RecO-mediated strand annealing reaction in the Δ*recA* background. This is consistent with the observation that the absence of RecA partially suppressed the need for RecO, but reduced the frequency of plasmid transformation ~200-fold in a Δ*dprA* background (Table 7).

The selection of recombination mediator will be depending on the substrate onto which RecA is to be loaded. Our data favours the hypothesis that SsbB participates in directing RecA loading via DprA, and that SsbA favours RecA recruitment via RecO during chromosomal transformation. If SsbB coats the incoming ssDNA strand, the SsbB·ssDNA, complex could antagonize the RecO mediated events and favours the utilization of DprA as mediator protein during plasmid transformation. SsbB could antagonize RecO mediator and interact with DprA for modulation of RecA. Conversely, if SsbA binds to the incoming ssDNA strand, the formed SsbA·ssDNA complex recruits RecO to form a ternary SsbA·RecO·ssDNA that facilitates RecO-mediated DNA strand annealing. However, when both SSB proteins co-assemble onto ssDNA, to form a SsbA·ssDNA·SsbB complex, both effectors (DprA or RecO) can facilitate RecA growth and DNA strand annealing.

Our data reveal a division of labour between the different RecA mediators (DprA and RecO) and the different SSB proteins (SsbB and SsbA). The SSB proteins play an active role for the selection of effector proteins (DprA or RecO), for chromosomal and plasmid transformation.

6. CONCLUSIONS

6. Conclusions:

1. A high-order RecA·ssDNA filaments, formed by assembly of RecA·dATP·Mg^{2+} onto ssDNA, is the active form of the RecA recombinase. RecA in the dATP bound form catalyses the formation of *jm* and the homologous pairing leads to strand exchange (*nc* products), but in the ATP bound form poorly characterize DNA strand exchange.

2. SsbA binds ssDNA with higher affinity and co-operativity, both in absence or presence of the metal ion, than SsbB. Both, SsbA and SsbB, have two-conserved mode of binding to ssDNA.

3. Crystal structure of the SsbB·ssDNA complex reveals that the SsbB tetramer has a similar structural features, than other SSBs, except few bases which faces into opposite direction in compare to SSB_{Eco}. AFM study reveals that SsbA and SsbB forms similar beaded structure onto ssDNA, albeit SsbA showed a higher binding affinity than SsbA.

4. SsbA and SsbB act to limit RecA nucleation onto SsbA- or SsbB-coated ssDNA. In a genetic search for potential accessory factor that overcome this kinetic barrier we have identify two mediators. The absence of RecO and DprA reduces chromosomal transformation by ~2- and 60-folds, respectively. However in the absence of both chromosomal transformation was completely abolish (~ 10^4 fold reduction).

5. In the presence of dATP, SsbA or SsbB limits RecA nucleation and filament growth onto ssDNA, albeit with different efficiency. In the strand exchange reaction SsbA could remove secondary structure more efficiently than the SsbB and facilitates the formation of *jm* and final *nc* products with higher efficiency than SsbB. In the presence of ATP, SsbA or SsbB blocks RecA nucleation and filament growth onto ssDNA. RecA-mediated DNA strand exchange is not observed in the presence of SsbA or SsbB. In the absence of ATP hydrolysis (presence of ATPγS), however, SsbA facilitates RecA-mediated DNA strand exchange.

6. In the presence of dATP, RecO alone does not significantly facilitate RecA filament growth onto ssDNA, and increases RecA-mediated accumulation of *nc* products during strand exchange. RecO facilitates RecA assembly onto SsbA- or SsbA·ssDNA·SsbB pre-coated ssDNA and enhances DNA strand exchange. In the presence of ATP, RecO facilitates RecA assembly onto ssDNA, but RecO cannot facilitate RecA-mediated recombination. RecO facilitates RecA assembly onto SsbA- or SsbA·ssDNA·SsbB pre-coated ssDNA, and facilitates RecA-mediated DNA strand exchange.

7. DprA binds ssDNA forming discrete blobs. The blob size increases with increasing protein concentration. DprA partially dislodged the SSB proteins from ssDNA. We proposed that DprA might distort the ssDNA and such effect facilitates SSB disassembly from the ssDNA.

8. In the presence of dATP, DprA does not significantly facilitate RecA assembly onto ssDNA, whereas alleviate the SsbB barrier on RecA filament growth. RecA

can assemble on the SsbB·ssDNA·DprA complex more efficiently than on SsbA·ssDNA·DprA complexes.

9. The recA accessory factors act to limit (SsbA, SsbB) and to faciliate (RecO, DprA) RecA activities, with RecO acting in concert with SsbA and DprA with SsbB.

10. RecO and DprA facilitate the spontaneous annealing of complementary DNA strands *in vitro*. Inactivation of RecO or DprA results ~30-fold ~40-fold reduction in plasmid transformation, respectively. Whereas, *ΔrecO Δ*dprA cells are blocked in plasmid transformation.

11. The SSB protein reduces spontaneous annealing of complementary ssDNAs. RecO or DprA by promoting bridging faciliatates the annealing complementary strand and preferentially alleviates the effect of their cognate SSB protein.

12. Absence of DprA and RecA results in ~200-fold reduction in plasmid transformation, whereas the absence of RecA overcomes the absence of RecO. We proposed that DprA plays a crucial role for plasmid transformation in absence of RecA.

7. SUMMARY IN SPANISH

7. Summary of Thesis (in Spanish)

1. Introducción:

La competencia natural es una etapa fisiológica en la que componentes de la maquinaria de captación, procesado del ADN y la translocación de una de las hebras (maquinaria de internalización del ADN) se ensamblan en uno de los polos de una bacteria de forma bacillar o en una posicion opesta al plano de division en una bacteria en forma de coco. Proteínas citosólicas de recombinación interaccionan con las priteínas de la maquinaria de de En Firmicutes esta maquinaria se une al ADN medioambiental, introduce un corte en una de las hebras y la degrada. La cadena intacta interacciona con las proteínas translocadoras. El ADN es internalizado como AND de hebra simple (ADNhs) y requiere protección frente a nucleasas citosólicas. Varias proteínas de recombinación, cuya síntesis también se induce durante la competencia natural interacionan con las proteínas de la maquinaria de internalización del ADN y se ensambaln en ese polo celular. El ADNhs interiorizado tienen diferentes destinos, y ello depende de la disponibilidad de homología en el genoma, sugiriendo que éstos destinos pueden ser consecutivos en lugar de simultaneaos. Si el sistema encuentra homología el ADNhs se ssDNA integrar en el genoma de la célula vía el intercambio de cadenas promovido por RecA (transformación cromosómica). En ausencia de homología las proteínas mediadoras facilitan la hibridación de las cadenas de ADNhs (transformación plasmídica).

B. subtilis, como la gran mayoría de células competentes, expresan dos proteínas tipo SSB, llamadas SsbA y SsbB. SsbA, con una longitud de 172 aminoácidos, presenta una alta identidad con SSB_{Eco}. Ambas, SsbA o SSB_{Eco} juega un papel principal en la replicación, reparación y recombinación del ADN. SssB es más proteína más corta (113 aminoácidos) y presenta alta identidad con SsbA (63%) en los primeros 106 amonoácidos. Ambas proteínas se inducen durante el proceso de competencia. Al contrario que SsbA, SsbB carece del extremo C-terminal ácidico y flexible. La cola ácida en la región C-terminal es responsable de la interacción con otras proteínas de replicación, recombinación y reparación. Las proteínas SSB actúan como guardianes del ADNhs internalizado y al mismo tiempo ejercen un efecto papel regulador limitando la nucleación y filamentación de RecA en el ADNhs. Las proteínas mediadoras alivian esta restricción y facilitan las actividades mediadas por RecA.

En nuestro estudio hemos caracterizado las actividades bioquímicas de los mediadores (RecO y DprA) asociados con SsbA y SsbB para la transformación cromosómica y plasmídica.

2. Objetivos:

1. Caracterización bioquímica de SsbA y SsbB de *B. subtilis*. La finidad de éstas por el ADNhs, los tipos de complejos que forman, y su estabilidad.

2. Papel de SsbA y SsbB en la nucleación y polimerización de RecA en ADNhs, y su contribución en el intercambio de cadenas llevado a cabo por RecA.

3. Identificación de los mediadores de recombinación genética: papel de RecO y DprA en la transformación cromosómica y plasmídica.

4. Papel de RecO como mediador de RecA: caracterización del (los) potenciales proteínas que trabajan concertadamente con RecO para facilitar la actividad de RecA durante la transformación cromosomal.

5. Caracterización bioquímica de RecO como proteína que anilla ADNhs durante la transformación plasmídica.

6. Caracterización de la unión a ADNhs de DprA de *B. subtilis* y su posible interacción con las proteínas SSBs

7. Papel de DprA como mediador de RecA en presencia de las proteínas SSBs durante la transformación cromosómica.

8. Caracterización bioquímica de la capacidad de DprA de anillar ADN de cadena sencilla complementario en presencia de las proteínas SSBs durante la transformación plasmídica.

9. Efecto del dATP y ATP en la nucleación y polimerización de RecA en un ADN de cadena sencilla recubierto por SsbA y/o SsbB.

10. Efecto del dATP, ATP or ATPγS en la reacción de intercambio de tres cadenas llevada a cabo por RecA.

3. Resultados y Discusión

La competencia es una etapa fisiológica donde la inducible maquinaria de procesado del DNA ambiental se une al él, lo procesan y translocan al interior de la bacteria diferenciada hacia la competencia natural. Las proteínas citosólicas tipo SSB, actuando como guardianes del ADNhs internalizado lo protegen de nucleasas y a su vez limitan en nucleado y la filamentacion de la recombinasa RecA en el ADNhs internalizado. SsbA, que es esencial para la proliferación bacteriana, se une a ADNhs con 8 veces mayor afinidad que SsbB, aunque ésta última es casi 6 veces más abundante que la primera.

La estructura de SsbB unida a su sustrato ha sido determinada. En este trabajo hemos demostrado que tienen una estructura similar a otras proteínas del tipo SSB aunque es un 33% más pequeña. Un tetramero de SsbB se une a ADNhs de dos maneras distintas: en el modo SSB_{35} sólo dos protomeros de la proteína interaccionan con el ADNhs y los envuelven, pero en el mode SSB_{65} los cuatro protomeros de SsbB estan envueltos por el ADNhs.

La inactivación de RecO y DprA reduce la transformación cromosomica caso 1000 veces, y a niveles comparables con la ausencia de RecA, sugiriendo que RecO y/o DprA podrían tener un papel como proteínas accesorias (mediadores). Las proteínas mediadoras tienen dos actividades: remueven las barreras impuestas por las proteínas tipo SSB en el ensamblado de RecA en el ADNhs, y ii) catalizan el anillado de secuencias homologas. El objetivo de las proteínas mediadoras en reducir la barrera impuesta por las proteínas SSB, facilitando su desalojo parcial del ADNhs y directamente contribuir al cargado de RecA en el ADNhs.

En la primera actividad las proteínas accesorias facilitan el proceso de transformación cromosómica. En presencia de dATP, RecO promueve parcialmente el desensamblado espontaneo de SsbA (ó SsbA y SsbB) y facilita el nucleamiento y el crecimiento del filamento de RecA en el ADNhs cubierto por las proteínas tipo SSB. RecO incrementa la frecuencia de recombinación llevada a cabo por RecA en ADNhs desnudo ó recubierto por proteínas tipo SSB. RecO no parece favorecer las actividades de RecA si el ADNhs esta recubierto por SsbB ó SSB_{SPP1}. En presencia de ATP, la nucleación de RecA en el ADNhs es pobre, comparado con la presencia de dATP, y es incapaz de catalizar el intercambio de cadenas. RecO facilitata el ensamblado de RecA en ADNhs, pero es incapaz de facilitar el proceso de recombinación mediado por RecA. RecO sólo puede facilitar ese proceso si el ADNhs esta recubierto por SsbA ó por ambas proteínas tipo SSB.

La proteína DprA también tiene actividad de mediador. En presencia de dATP, DprA facilita el ensamblado de RecA en el ADNhs. DprA reduce la barrera impuesta por las proteínas del tipo SSB, las desplaza parcialmente y promueve el cargado de RecA en el ADNhs. RecA se ensambla con mayor eficiencia en los complejos SsbB·ssDNA·DprA que en los complejos SsbA·ssDNA·DprA.

En la segunda actividad las proteínas accesorias facilitan el proceso de transformación plasmídica. La inactivación de RecO o DprA reduce la transformación plasmídica 30 o 40 veces, pero la inactivación de ambas reduce la transformación plasmídica ~ 1000 veces. Las proteínas mediadoras son capaces de facilitar en apareamiento espontaneo de cadenas homologas recubietas por su proteína tipo SSB especifica. Las proteínas SsbA y SsbB límitan el apareaminto espontaneo de ADNhs complementarias. Sin embargo el mediador desaloja parcialme a la proteína SSB de ssDNA y acerca cadenas de ADNhs al generar puentes entre ambas hebras del ADN. RecO interacciona específicamente con SsbA y facilita su desplazamiento parcial. El la región de ADNhs desnudo puede aparearse si encuentra su región complementaria: éste proceso está faciliatado por RecO al ponerlas en proximidad distintas regiones del ADNhs. Las mismas actividade fueron demostradas para DprA, pero aquí su compañero de tarea en SsbB. DprA facilita el apareamiento de regiones homólogas cubiertas por SsbB.

La ausencia de RecA suprime el requerimiento de RecO transformación plasmídica pero es incapaz de suprimir el requerimiento de DprA, sugiriendo que DprA juega un papel clave en transformación plasmídica. Nuestros datos revelan una división del trabajo entre los diferentes mediadores (DprA y RecO) y las diferentes proteínas SSB (SsbB y SsbA). RecO y DprA tienen especificidad por SabA ó SabB, respectivamente. Postulamos que las proteínas SSB desempeñan un papel activo en la selección de las proteínas efectoras (DprA o RecO), para la transformación cromosómico y plasmídico.

4. Conclusiones:

1. Los filamentos formados por el ensamblado de RecA unida a dATP en el ADNhs llevan a cabo una búsqueda de regiones de ADN homólogas. La localización de estas regiones de ADN homólogo da lugar a la formación de moléculas anilladas (*jm*) y su intercambio con el ADN homólogo permite el intercambio de cadenas (*nc*). Ëstos procesosos no tienen lugar cuando el dATP es reeplazado por elATP. Existen proteínas accesorias que actúan para limitar (SsbA, SsbB) o facilitar (RecO, DprA) el ensamblado de RecA en el ADNhs. RecO actúa de forma concertada con SsbA y DprA con SsbB.

2. SsbA une ADN de cadena sencilla con alta afinidad y de forma cooperativa tanto en presencia como en ausencia de iones metálicos en comparación con SsbB. Tanto SsbA como SsbB tienen dos modos conservados de unión a ADNhs.

3. El cristal del complejo formado por SsbB y el ADNhs revela que el tetrámero de SsbB tiene características similares a otras SSBs. Estudios de AFM revelan que SsbA y SsbB forman estructuras de collar de perla en un ADNhs circular, con SsbA presentando mayor afinidad de unión que SsbB.

4. RecA hidroliza dATP de manera más eficiente que ATP. En presencia de dATP, SsbA o SsbB limitan la nucleación y el crecimiento del filamento de RecA en el ADN de cadena sencilla aunque con diferente eficiencia. En la reacción de intercambio de cadenas, SsbA puede eliminar estructuras secundarias más eficientemente que SsbB y, además, facilita la formación de moleculas anilladas (*jm*) y producto final (*nc*) con mayor eficiencia que SsbB. En presencia de ATP, SsbA o SsbB bloquean la nucleación y el crecimiento del filamento de RecA en ADN de cadena sencilla. No se observa el intercambio de cadenas mediado por RecA en presencia de SsbA o SsbB. Aunque en ausencia de hidrólisis de ATP (presencia de ATPγS), SsbA facilita el intercambio de cadenas mediado por RecA.

5. En los mutantes Δ*recO* y Δ*dprA* se reduce ~2- y 60- veces, respectivamente, la transformación cromosomal. Sin embargo, en la cepa doble mutante Δ*recO* Δ *dprA* la transformación cromosomal está completamente bloqueada (se reduce ~ 10^4 veces).

6. En presencia de dATP, RecO por sí sola no facilita la filamentación de RecA en el ADN de cadena sencilla y pero aumenta la acumulación de productos finales (nc) llevada a cabo por RecA durante el intercambio de cadenas. RecO facilita el ensamblado de RecA en el ADN de cadena sencilla recubierto por SsbA o SsbA y SsbB- y facilita el inercambio de cadenas de ADN. En presencia de ATP, RecO facilita el ensamblado de RecA en el ADN de cadena sencilla y no facilita la recombinación mediada por RecA. RecO facilita el ensamblado de RecA en el ADNhs recubierto por SsbA or SsbA y SsbB, y el intercambio de cadenas llevado a cabo por RecA.

7. La inactivación de RecO da lugar a una reducción de ~30 veces en la frecuencia de transformación plasmídica. RecO favorece el anillado de moléculas complementarias de ADN de cadena sencilla recubiertas por SsbA pero no lo hace cuando SsbB, SsbB* o SSB_{SPP1} están recubriendo el ADNhs. RecO actúa como un puente facilitando el anillamiento de moléculas de ADN de cadena secilla complementarias.

8. DprA une ADNhs formando bolas de diferente tamaño. El tamaño de éstas aumenta cuando aumenta la concentración de proteína. DprA desplaza parcialmente a las proteínas SSBs del ADNhs. Nosotros proponemos que DprA podría distorsionar el ADN de cadena sencilla y esto facilitaría el desensamblado de SSB.

9. En presencia de dATP, DprA no facilita marcadamente el ensamblado de RecA en el ADN de cadena sencilla, sin embargo alivia la barrera producida por la presencia de SsbB en el crecimiento del filamento de RecA. RecA puede unirse al complejo formado por SsbB·ssDNA·DprA de forma más eficiente que al complejo SsbA·ssDNA·DprA

10. La inactivación de DprA da lugar a una reducción de ~40 veces de la transformación plasmídica. Sin embargo, el mutante *ΔrecO ΔdprA* está totalmente bloqueado en transformación plasmídica. Las proteínas SSBs reducen el anillamiento espontáneo de moléculas de ADN de cadena sencilla complementarias. DprA facilita el anillamiento de cadenas complementarias y alivia el efecto inhibitorio de SsbB en la reacción.

11. La ausencia de las proteínas DprA y RecA da lugar a una reducción de ~200 veces de la transformación plasmídica mientras que se reduce solo ~2 veces en el doble mutante *ΔrecO ΔrecA*. DprA juega un papel fundamental en la transformación plasmídica en ausencia de RecA

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9. APPENDIX